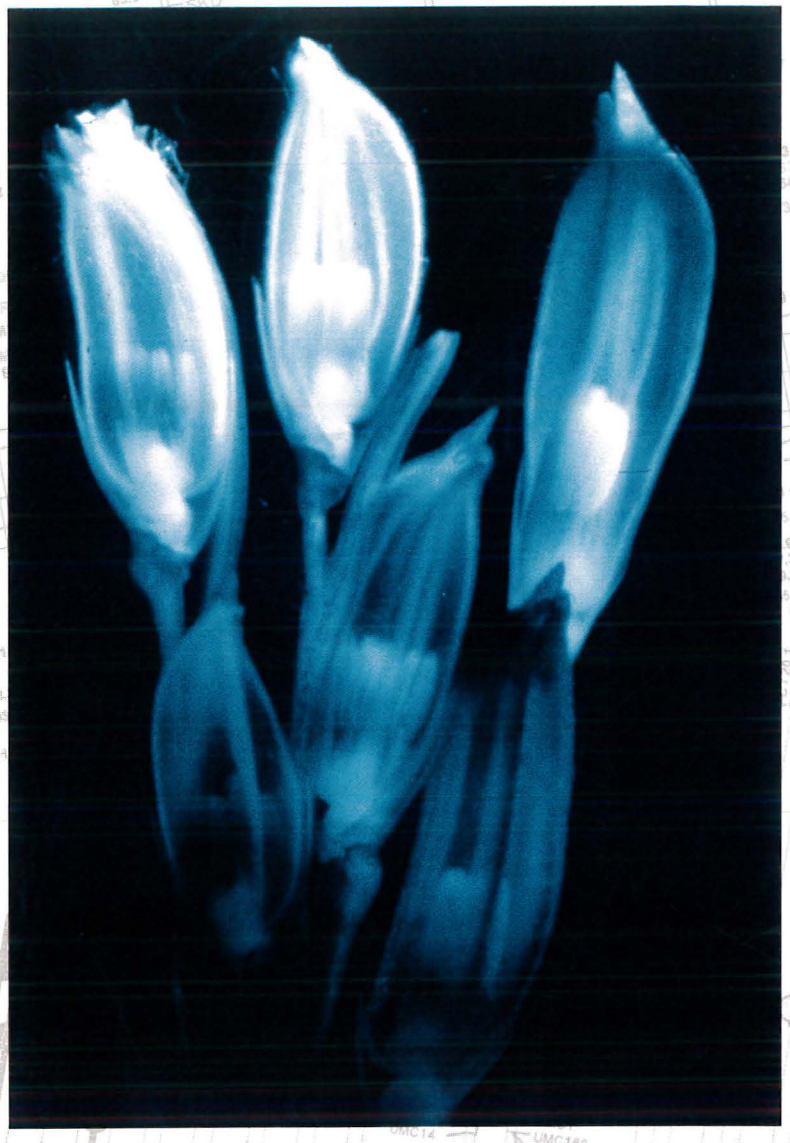

Cooperation in plant genomics at CIRAD

June 1998
CIRAD

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The Centre de coopération internationale en recherche agronomique pour le développement (CIRAD) is a French scientific organization specializing in development-oriented agricultural research for the tropics and subtropics. It is a state-owned body and it was established in 1984 following the consolidation of French agricultural, veterinary, forestry, and food technology research organizations for the tropics and subtropics.

CIRAD's mission is to contribute to the economic development of these regions through research, experiments, training, and dissemination of scientific and technical information.

The Centre employs 1800 persons, including 900 senior staff, who work in more than 50 countries. Its budget amounts to approximately 1 billion French francs, more than half of which is derived from public funds.

CIRAD is made up of seven departments: CIRAD-CA (annual crops), CIRAD-CP (tree crops), CIRAD-FLHOR (fruit and horticultural crops), CIRAD-EMVT (animal production and veterinary medicine), CIRAD-Forêt (forestry), CIRAD-TERA (territories, environment and people), and CIRAD-AMIS (advanced methods for innovation in science). CIRAD operates through its own research centres, national agricultural research systems, or development projects.

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Introduction

Plant genomics at CIRAD is aimed at assisting breeding programs. In accordance with its mandate, CIRAD has undertaken varietal improvement for a wide range of crops and a large array of environments. The essential basis is a rational use of genetic diversity available from germplasm collections and the refinement of gene incorporation or introgression methodologies through accurate genome mapping, marker-assisted selection and/or cloning and genetic transformation. The strength of these activities rests on efficient collaborations with partners from the South, in both the public and private sectors, and integrated research on stations in the tropics.

In many cases, these efforts are being coupled with complementary inputs from ORSTOM (Institut français de recherche scientifique pour le développement en coopération, France). They are also strengthened by collaborations with the French INRA (Institut national de la recherche agronomique) and CNRS (Centre national de la recherche scientifique) and support from the French Génoscope (national sequencing center). The research is often conducted within the frame of European projects.

Laboratories in Montpellier serve as a basis for innovative research, for adaptation before effective transfer to the research stations overseas and for training.

Germplasm survey and localization of useful genes

Molecular analysis of genetic diversity

CIRAD has had a pioneering role in the use of isozymes for the survey of genetic diversity among tropical plants in the early 1980s. Similar efforts have been intensified on several crops with the advent of molecular markers in the late 1980s; they permitted a better coverage of the genome, including its cytoplasmic components, and a linkage to genetic mapping. Such studies are being used for the constitution of core collections, often within a frame of international collaborations.

A number of tropical crops are derived from interspecific hybrids, both of natural and artificial origin. Genome dosage, interspecific recombination and gene introgression are often the key to quality materials and breeding efficiency. CIRAD has developed DNA *in situ* hybridization on plant chromosomes in order to study these aspects in particular crops such as sugarcane and banana-plantain. This is complemented with the study of repetitive sequences in the genome that may bear species- or genus-specificity.

Genome mapping and tagging of useful genes

The unlimited number of tags offered by molecular markers is used for building genome maps and targeting factors involved in traits of agricultural interest. Original saturated maps have been developed for banana, cocoa, eucalyptus, rubber tree, sorghum and sugarcane. This research is connected to field experiments in the tropics and under laboratory controlled conditions for dissecting the genetic control of the major breeding target traits. A major effort is directed towards studying the resistance to biotic stresses, in connection with molecular analyses of the pest and pathogen diversity also performed at CIRAD. Depending on the genetic control of the traits, these studies evolve more or less rapidly towards fine mapping and gene cloning. In the most advanced cases, genetic analysis is relayed by physical mapping using BAC libraries within collaborative projects.

Rice with its small and simple genome is being used as a model for the integration of genomic research and application to other grasses or other monocots. Genetic resources are being studied in detail, the genome is being analyzed as a central resource for comparative mapping, genes contributing to specific traits are being tagged, genetic stocks are being produced by transposon

mutation in view of gene cloning and transgenic plants are being used for promoter evaluation. Links are ensured with INRA for comparable studies on *Arabidopsis* to serve as a model for dicots.

Somatic embryogenesis and genetic transformation

Micropropagation is a very efficient tool for propagation of a genetic material obtained by plant breeders. This is of a special interest in this case of ligneous species predominantly allogamous where the "elite plants" cannot be multiplied through seeds or even through classical horticulture. Somatic embryogenesis, especially with cell suspensions, offers a powerful tool for multiplication. CIRAD has been involved for many years in that field of research for two main purposes i) mastering the whole process for a true-to-type multiplication, taking into account the risk of somaclonal variation, ii) using embryogenic cell suspensions as targets for genetic transformation. All studies have been carried out with the help of histological surveys from the chosen explant to the regeneration of vitroplantlets. Field experiments are also going on to verify the conformity with the mother plant (e.g. banana) or to validate the utility of the process (e.g. rubber tree).

Genetic transformation experiments have started on several plants: banana, coffee, cotton and rice. These systems are being used for testing promoter and gene action. Besides the efforts aimed at cloning genes from the target plants, CIRAD has developed activities using the bacteria *Bacillus thuringiensis (Bt)* to try and control important pests. A particular effort has been placed on screening toxins (and genes) available from the many different strains of *Bt* and constructions especially adapted for genetic transformation of monocots or dicots have been made up.

Supporting research

Since pests and diseases are the main cause for yield losses, CIRAD and ORSTOM are taking advantage of their site in Montpellier out of the production areas for assembling collections of parasitic agents and conducting surveys of their genetic diversity. These studies lead to a better understanding of epidemics, which enables limiting their spread through quarantine and refining the breeding of resistant varieties.

Germplasm survey and localization of useful genes

Banana and plantain

Banana (including plantain) belong to the genus *Musa* of the Musaceae family (monocot). They originate from Southeast Asia. Wild bananas are diploid with sexual reproduction; cultivated bananas are mostly polyploid (triploid and tetraploid), natural hybrids between two species, *M. acuminata* (genome A) and *M. balbisiana* (genome B). The main part of the production comes from sterile triploids that are propagated vegetatively. One of the main selection criteria is the resistance to the black leaf streak disease (BLSD) caused by the fungus *Mycosphaerella fijiensis.* CIRAD has a unique expertise in banana germplasm characterization, molecular analysis of genetic diversity, genome mapping and evaluation in relation to BLSD.

Molecular analysis of genetic diversity

RFLPs were used as markers to determine the transmission of cytoplasmic DNA in diploid banana crosses. Progenies from controlled crosses were studied with heterologous cytoplasmic probes. This analysis provided evidence for a strong bias towards maternal transmission of chloroplast DNA and paternal transmission of mitochondrial DNA in *Musa acuminata.* These results suggest the existence of two separate mechanisms of organelle transmission and selection. Knowledge of the organelle mode of inheritance constitutes an important point for phylogeny analyses in bananas and may offer a powerful tool to confirm hybrid origins.

RFLPs were also used to analyze the genetic diversity of 160 diploid bananas: 70 seminiferous clones (wild bananas) of the species *Musa acuminata, M. balbisiana* and *M. schizocarpa,* 90 parthenocarpic diploid as well as 150 polyploid clones (cultivars) were studied using 30 mapped single copy nuclear probes and 10 chloroplast and mitochondrial probes. Alleles specific to the three species studied were found. *M. acuminata* was the most polymorphic. *M. schizocarpa* appeared to be very similar to *M. acuminata* subsp. *banksii,* and *M. balbisiana* was more divergent. All the diploid parthenocarpic bananas contain *M. acuminata* alleles. This confirms the involvement of *M. acuminata* in the origin of parthenocarpy. Some cultivars were proven to be hybrids between *M. acuminata* and *M. schizocarpa.* "Starchy" cultivars were found to be closely associated with *M. acuminata* subsp. *banksii,* whereas "sweet" cultivars were close to *M. acuminata* subsp. *malaccensis.*

The two main basic genomes (A representing *M. acuminata,* B representing *M. balbisiana*) could be differentiated by genomic *in situ* hybridization. Repetitive sequences were investigated, in particular for their potential genome specificity. Different SINE-like and LINE-like repetitive sequences could be reported: Copia-like and Alu-like elements, of which a sequence family of species-specific repetitive elements, Brep 1, is of special interest. The Brep 1 sequence family is distributed throughout the Musaceae with various copy numbers. The two species *M. acuminata* and *M. schizocarpa* carry the highest copy numbers in contrast to *M. balbisiana* and the tested representatives of different other sections. PCR primers were defined in the core consensus sequence for specific amplifications, which allow representatives of this sequence family to be easily detected in wild and cultivated banana clones. Sequence data were analyzed and hypotheses on the evolution of banana cultivars from the wild-type banana clones could be inferred.

The triploid (AAA) Cavendish group appeared related to *M. acuminata* subsp. *errans* and *M. acuminata* subsp. *malaccensis* on the basis of the cytosolic RFLP probes whereas RFLP data from the nuclear genome did not allow for any strong association with either subspecies. In spite of the high number of nuclear probes used in this study, it was not possible to relate this triploid clone to any diploid wild-type subspecies. Repeated sequences, on the other hand, form a large portion of the genome. In this particular case, it was possible to link the triploid cultivar to the

zebrina subsp. This leads to the hypothesis that three *M. acuminata* subspecies are included in the complex genome formula of Grand Nain cultivar: *errans* subsp., *malaccensis* subsp. and *zebrina* subsp.

A method, based on competitive reverse transcription-polymerase chain reaction, using Brep 1 sequence information, could be used to determine the copy number of nuclear repetitive elements. The reliability of this method was investigated on crude leaf-extracted total DNA. Copy number assays of repetitive elements using this method clearly distinguished between the two banana subspecies investigated: *M. acuminata* subsp. *banskii* and *M. acuminata* subsp. *malaccensis.*

A powerful marker system can be generated using microsatellite sites- and locus-specific PCR: STMS. In banana and plantain this method allows for the identification of A- and B-genome-specific bands and the classification of *Musa* (sub)species and cultivars. STMS discrimination potential was explored using 9 microsatellite primer pairs. Genetic relationships were examined among 59 genotypes of wild or cultivated accessions of diploid *Musa acuminata.* The organization of the subspecies was confirmed and some clone relationships were clarified.

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Genome mapping and tagging of useful genes

A composite map has been constructed (unpublished results) from two mapping populations (200 individuals) at a LOD score of 4.75 (Figure 4). This composite map covers 1227 cM and links 373 isozyme, microsatellite, RFLP, RAPD and AFLP markers in 11 linkage groups. The mean linkage distance is 3 cM. Up to 36% of all the markers tested show important segregation distortions. A complete comprehensive map of *Musa* spp. will be attainable through the comparison of several maps based on parents with different karyotypes. A subset of co-dominant markers, spaced 10-20 cM apart, is being selected to construct a frame map which could be saturated easily using AFLP, as an example. Eventually this "anchor marker set" will be distributed to interested researchers in a cooperative research effort.

Forty-five STMS from CIRAD have been published in the EMBL (European Molecular Biology Laboratory, Germany) database and are accessible via Internet (http://www.ebi.ac.uk; EMBL accessions X87258 to X87265, X90740 to X90750 and Z85950 to Z85977). A molecular database, Tropgene, is being constructed at CIRAD, harboring all our data (ACeDB algorithm). The different maps eventually produced may be compared through the relative localization of the same anchor markers. The comparative data resulting from these efforts should be used to map translocation breakpoints in order to produce a *Musa* consensus or core map.

The QTL mapping of resistance to BLSD is being continued within the international banana improvement program in the outstation in the French West Indies (Guadeloupe) and the Regional Research Center for Banana and Plantain (CRBP) in Cameroon.

Selected publications

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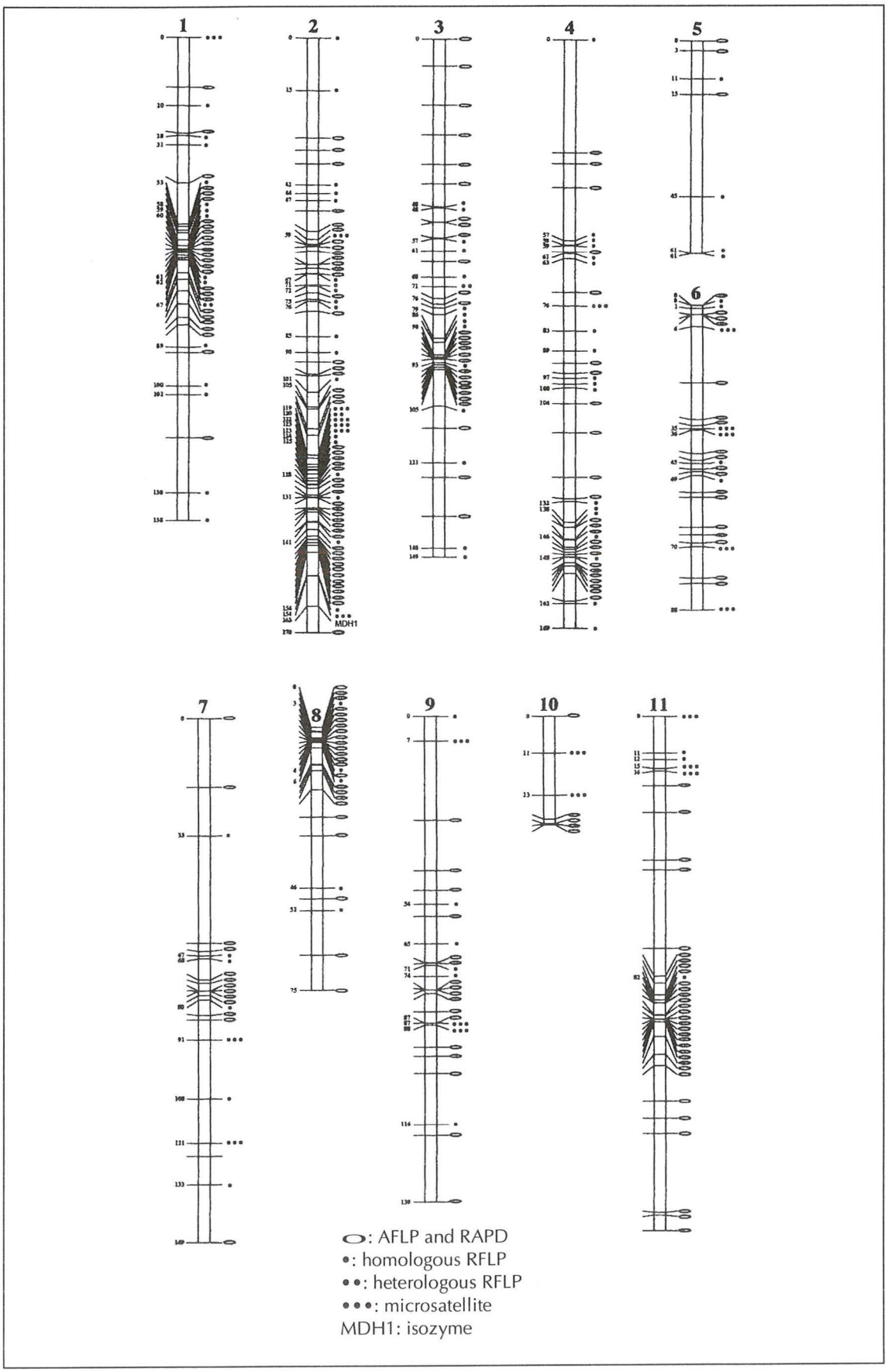

Figure 4. Core map of banana.

Cocoa

Theobroma cacao L. (2n = 2x = 20) is a Sterculiaceae and is native to central and South America. Its genome is small (0.4 pg/1C). Three main genetic groups can be distinguished: Criollo, Forastero and their hybrid form, Trinitario. Its domestication dates back to the Maya civilizations. Although the first place of domestication was located in central America, Cheesman (1944) considered that the center of origin of *T. cacao* is in the Upper Amazon near the Ecuadorian Andes because of the large diversity encountered in this region. Cocoa improvement rests on hybrids between clones of distinct morphogeographic groups. Molecular markers are being applied to characterize genetic resources and refine *T. cacao* classification. They are also used for analyzing the genetic basis of traits of interest through genome mapping with the view to developing marker-assisted selection activities.

Molecular analysis of genetic diversity

Genetic diversity of *T. cacao* was studied on a sample of 350 genotypes belonging to the different morphogeographic groups. Several kinds of molecular markers were used for these analyses. RFLPs were monitored using probes obtained from a genomic and a cDNA library, as well as cytoplasmic (chloroplastic and mitochondrial) heterologous probes and ribosomal DNA probes. Other kinds of markers such as RAPDs and microsatellites were applied for the study of a smaller sample.

The analyses showed a clear differentiation between Criollo and Forastero clones. The Trinitario accessions are widespread between these two groups. Several populations can be distinguished within the Forastero group: the Forastero from Upper Amazon which display the largest diversity, the Forastero from Lower Amazon and the Forastero from French Guiana.

The Criollo group, which gives the finest chocolate, was more thoroughly studied. Several hypotheses have been formulated for the origin of this group. Our results clearly show that Criollo were differentiated independently from Forastero during the evolution of the species and that they do not originate from the Forastero group.

Criollo cacao was the first domesticated cacao. It is now almost completely replaced in the plantations by Trinitario hybrids, which are more vigorous. The analyses of remote Criollo types collected in Venezuela and Mexico demonstrated the nearly unique origin of this type with diverse morphological forms associated with a narrow molecular diversity. The present Criollo varieties correspond to this ancestral Criollo type more or less introgressed by Forastero genes.

The differentiation between Criollo and Forastero has been also observed for the size of the genome, with a the smaller genome in the Criollo forms.

A database, Tropgene, has been established which gathers all the molecular information on the samples studied.

Selected publications

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Genome mapping and tagging of useful genes

A high density linkage map of *T. cacao* has been established from a population of 182 individuals derived from a cross of two heterozygous plants: a Trinitario and a Forastero (Figure 3). The map comprises 419 loci linked in 10 groups which correspond to the ten chromosomes of *T. cacao.* These loci correspond to 5 isozymes, 172 RFLP probes, 30 RAPD and 191 AFLP markers and 15 microsatellites. The length of the map is 868 cM with a 2.1 cM average distance between two markers.

This map is now used to study the basic components of agronomic characters and to locate loci (QTLs) affecting traits of interest such as disease resistance, yield factors and quality traits.

The first results concern the resistance to *Phytophthora palmivora,* the causal agent of black pod disease, which is spread worldwide and is responsible for important yield losses. A population of 144 individuals located at Bingerville (Côte d'Ivoire) was analyzed using AFLP and microsatellites. Several QTLs were observed: two regions of the genome, similar in the two parents, explained 36% of the variability of the percentage of infected pods. Two QTLs explaining 23% of the variation for yield have also been revealed by this study.

Three progenies located in Cameroon were mapped by use of RFLPs, AFLPs and microsatellites to study resistance to *Phytophthora megakarya.*

Current QTL analyses developed at CIRAD also deal with other traits of interest such as various productivity factors and quality traits.

Present efforts also aim at developing additional PCR markers such as microsatellites and CAPS in order to facilitate marker use in tropical countries for clonal identification and marker-assisted selection.

Selected publications

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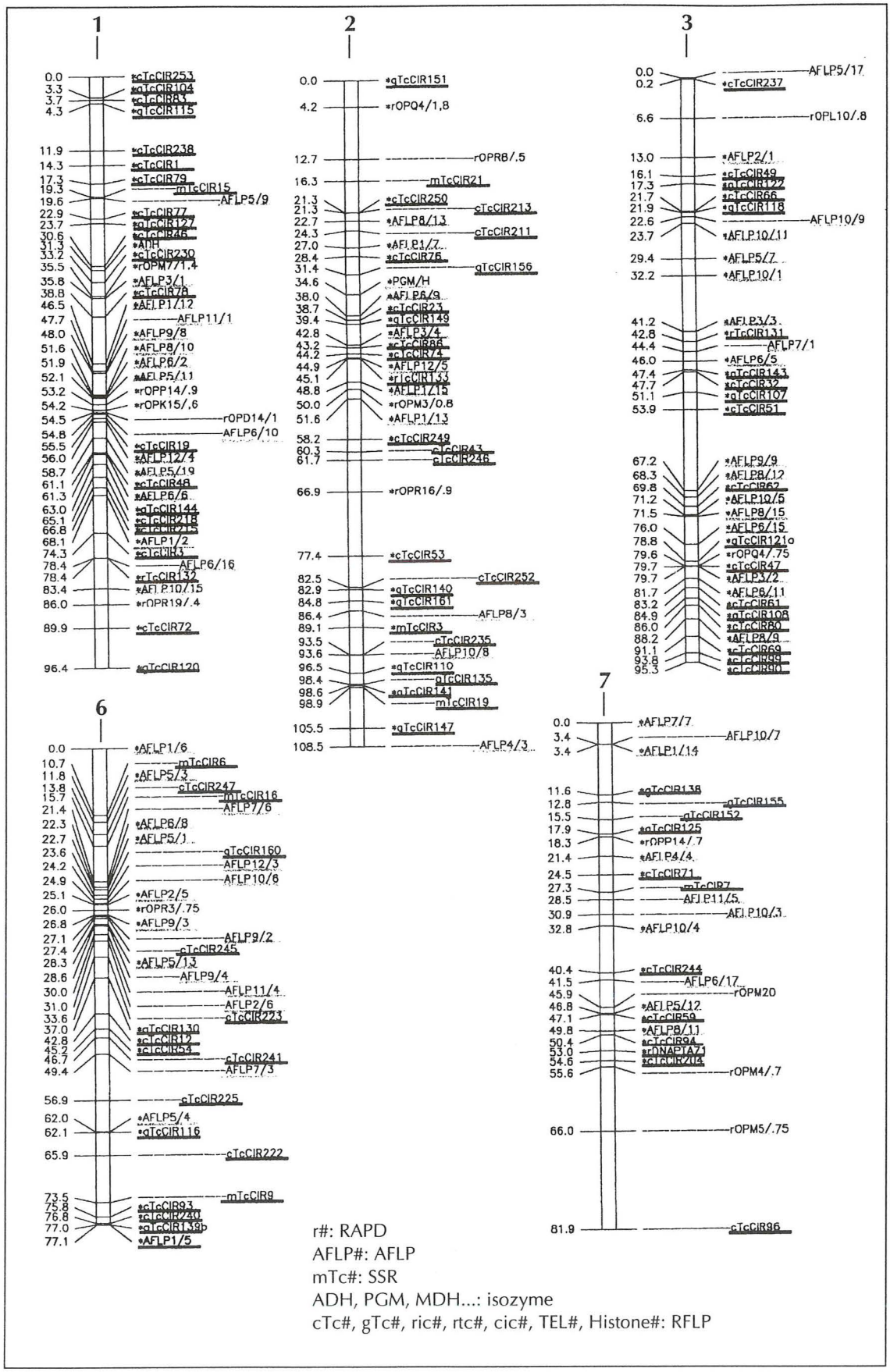

Figure 3. Genetic map of cocoa.

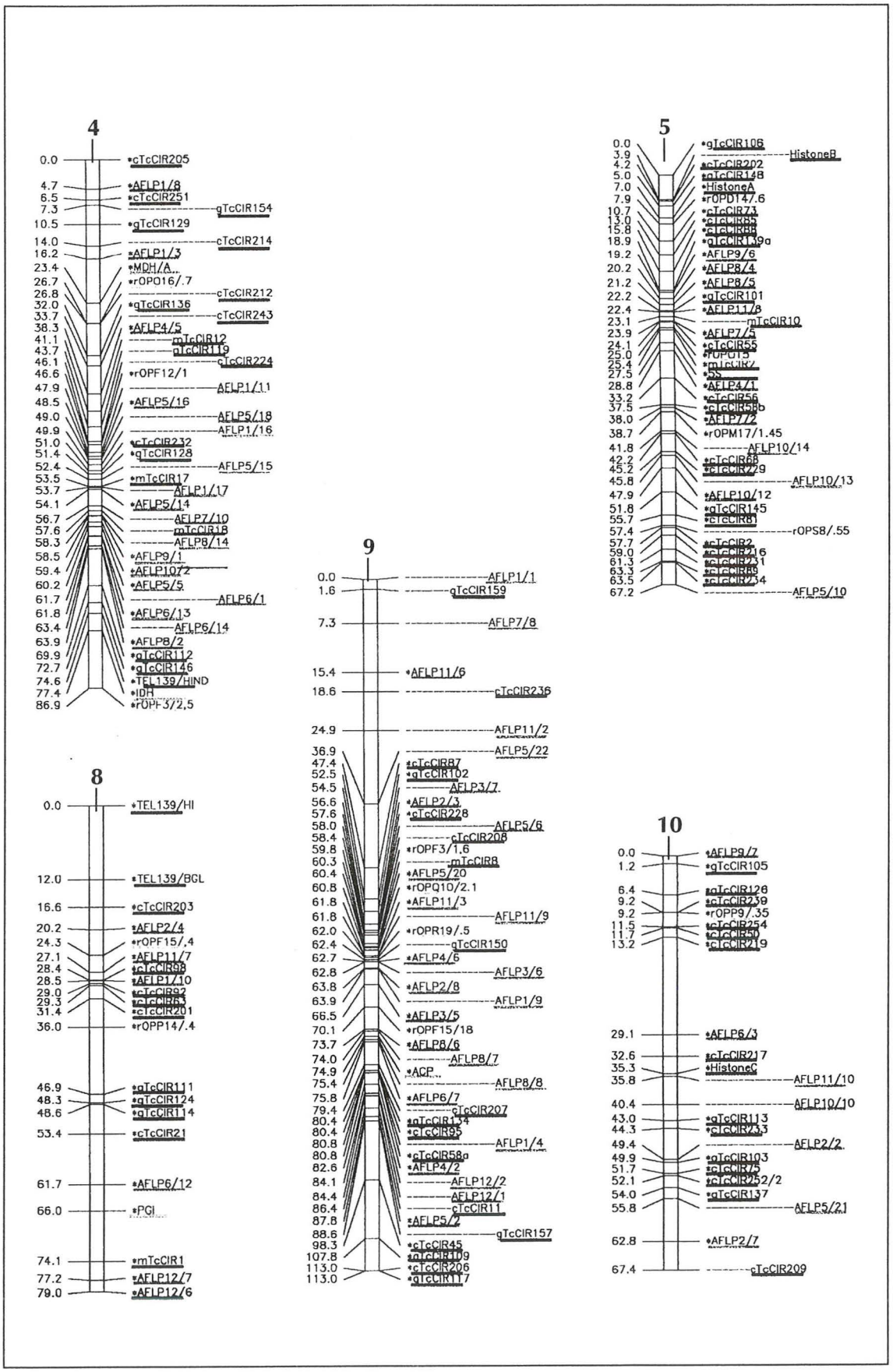

Figure 3. Continued

Coconut palm

Coconut is a pantropical crop, cultivated mostly in coastal areas. It is propagated via seeds. The species *Cocos nucifera* has no wild relatives. Most coconut palms belong to the "Tall" type which is allogamous, while the "Dwarf" is essentially autogamous, and may be distinguished by its reduced growth habits and by its nuts which are smaller and generally brightly colored. Among Talls, diversity studies using morphological and leaf polyphenol characters have made it possible to distinguish populations originating from Indian and West African coconuts and those from Southeast Asia and the Pacific Ocean.

Molecular analysis of genetic diversity

A first study of the molecular diversity in coconut has been conducted with over 100 individual palms. It was recently extended to 289 palms, representative of 26 Tall and 16 Dwarf cultivars from the major coconut cultivation areas. Twenty nuclear probes from oil palm, rice, maize, and coconut and 1 cytoplasmic probe from wheat were used in combination with 4 restriction enzymes. Factor analysis of correspondence was used to summarize the structure of the variation observed at the molecular level. These results, along with geographical and historical considerations, confirmed the identification of two main groups of Tall coconuts, originating respectively from a large area including Southeast Asia and the Pacific Ocean and from the Indian subcontinent. Cultivars from East Africa and from the Andamans share markers of both groups whereas Panama Tall appears to be derived from the first one. All Dwarfs (except for Niu Leka) form a very homogeneous group related to the first group of Talls. This provides a new insight into the evolution of coconut and opens perspectives for the use of molecular markers for coconut germplasm management and utilization.

Selected publications

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Eucalyptus

Genus *Eucalyptus* encompasses more than 550 species classified into eight subgenera. Most cultivated species belong to subgenus *Symphyomyrtus.* They are diploid and preferentially allogamous. Many interspecific cross combinations are possible; CIRAD is concentrating genome analysis efforts using an interspecific family between the two species *E. urophylla* and *E. grandis.*

Molecular analysis of genetic diversity

The development of molecular techniques can be efficiently incorporated into interspecific breeding programs in the *Eucalyptus* genus for the tropical region of Congo. Within the frame of the reciprocal recurrent selection scheme developed in 1989 by CIRAD on *Eucalyptus,* RAPD assays were performed to assess the genetic diversity in the two species *E. urophylla* and *E. grandis.* The molecular markers were split into two parts: the specific markers (present with different frequencies in the two species) and the common markers (present with similar frequencies in the two species). The study analyzed the structure of genetic diversity within and between the two species of *Eucalyptus.* Different genetic distances were worked out for use in prediction equations of the individual tree trunk volume of hybrids at 38 months. Each distance was expressed as the sum of the general genetic distance and the specific genetic distance. The general genetic distance based on bands with double presence or double absence appeared as an interesting co-variate to use in a factor regression model. Through this model the distance calculated between species explained the general combining ability (GCA) and the specific combining ability (SCA) of the phenotypic character with a global coefficient of determination of 81.6%.

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Genome mapping and tagging of useful genes

Two single-tree linkage maps were constructed for *E.* urophylla and *E. grandis,* based on the segregation of 480 RAPD markers in an F_1 interspecific progeny. A mixture of three types of single-locus segregations was observed: 244 1:1 female, 211 1:1 male and 25 markers common to both, segregating 3:1. Markers segregating in the 1:1 ratio (testcross loci) were used to establish separate maternal and paternal maps, while markers segregating in the 3:1 ratio were used to identify homology between linkage groups of the two maps. An average of 2.8 polymorphic loci were mapped for each arbitrary ten-mer primer used in the polymerase chain reaction. The mean interval between framework markers on the maps was 14 cM. The maps comprised 269 and 236 markers covering 1331 cM and 1415 cM in 11 linkage groups, for *E. urophylla* (2n = 2x = 22) and *E. grandis* (2n = 2x = 22), respectively. A comparative mapping analysis with two other

E. urophylla and *E. grandis* linkage maps showed that RAPDs could be reliable markers for establishing a consensus species-map. RAPDs markers were automatically and quantitatively scored with an imaging analyzer. They were classified into four categories based on their optical density. A fragment intensity threshold is proposed to optimize the selection of reliable RAPD markers for future mapping experiments.

The objective of another study was to use RAPDs to determine the genetic location and effects of genomic regions controlling wood density, stem growth and stem form in two species of *Eucalyptus.* Two hundred F_1 trees generated from an interspecific cross *E. urophylla* x *E. grandis* between two elite trees were used. Genetic maps were constructed for each parent with markers segregating in the 1:1 ratio in F_1 progeny. A total of 86 and 92 markers distributed among 11 linkage groups covered 1295 cM and 1312 cM for the *E. urophylla* and *E. grandis* parents, respectively. Traits were measured three times up to selection age (38 months). The magnitude of the phenotypic variation explained by the joint action of the segregating quantitative trait alleles indicated that genetic factors of large effects were involved in the control of the characters studied. Several regions controlling part of the variation were identified by interval mapping. Some regions of the genome exerted effects on more than one trait, providing a genetic explanation for at least part of the correlation between the traits. Based on an age by age analysis, a partial stability of QTLs expression was observed with 68% of the QTLs being expressed at two ages and 32% being age-specific. None was detected for all three ages. Taking advantage of repeated measurements on the same material across different ages, we investigated with a maximum statistical power the effect of marker genotype on traits, age and QTL × age interaction effects being removed. A two-way analysis of variance made it possible to detect significant marker-trait associations over the period studied. Most of them had already been detected in the annual analysis. This result is very encouraging for the application of marker information to early selection of hybrid trees that will be vegetatively propagated for the production of clonal varieties.

The feasibility of marker-assisted selection in forest tree breeding is being considered, in particular on the basis of a maritime pine and a *Eucalyptus* breeding program. The main difficulty is the expected absence of linkage disequilibrium between markers and QTL in large random mating populations. A decrease in costs and automation of the RAPD technique can make it possible to construct single-tree maps for every individual of an elite breeding population: an extreme alternative to dealing with linkage equilibrium. Effective mapping and QTL detection strategies using existing pedigrees in forestry breeding programs (half-sib and full-sib progenies) can be identified and genetic gains and costs associated with the use of molecular markers can be compared to other schemes that aim at a similar selection efficiency.

Selected publications

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Verhaegen D., Plomion C., Gion J.M., Poitel M., Costa P., Kremer A., 1997. Quantitative trait dissection analysis in *Eucalyptus* using RAPD markers. 1. Detection of QTLs in interspecific hybrid progeny, stability of QTL expression across different ages. Theor. Appl. Genet. 95:597-608.

Maize

CIRAD has been working on maize resistance to various viruses in Réunion, where natural pressure is high. Insect vector populations have been developed for accurate screening techniques. The work has focussed on the genetic analysis of the resistance and its transfer to alien elite materials. Genome mapping experiments have been undertaken since 1993 in collaboration with CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo, Mexico).

Genome mapping and tagging of useful genes

Maize streak virus (MSV), maize mosaic virus (MMV) and maize stripe virus (MStV) diseases can cause significant grain yield reduction in maize. In order to reach a better knowledge of the genetic control of the resistance to each disease and thus to improve the selection schemes in current use, an RFLP mapping study was performed.

D211 and L61, two completely resistant lines to MSV from Réunion Island, were each crossed to B73, completely susceptible, to form two F_2 segregating populations and their derived $F_{2:4}$ families (165 and 191 families respectively). These families were tested for MSV resistance under artificial infestation in two different environments: Harare, Zimbabwe, and Saint-Pierre, Réunion.

MMV resistance was mapped in an F_2 population derived from the cross between Hi40, resistant, and B73, susceptible. One hundred and five $F_{2:4}$ families were scored under insect proof tunnels after artificial infestation in Saint-Pierre.

In all the trials, six disease assessments were made on 15 to 24 plants per plot and two replications. Around 124 RFLP markers were mapped depending on the population. Composite interval mapping was used on the AUDPC (area under the disease progress curve) variable in Harare for MSV resistance and in Réunion for MMV resistance.

Resistance to MSV appeared as controlled by one major QTL (or cluster of QTLs) on chromosome 1 (QTLS1), explaining 69% of the variation, and at least one minor one on chromosome 10. QTLS1 had a predominantly additive effect. Analyses of other available data will allow us to test the stability of these QTLs across environments and germplasm.

Two QTLs for MMV resistance were detected, one on chromosome 6 (QTLM6), and the other on chromosome 3 (QTLM3) which may be a cluster of two or three loci. They explained 6% and 68% of the phenotypic variance, respectively. The genotypic effect at QTLM3 was predominantly additive, which should simplify selection for this genomic region.

This work will be extended to the mapping of MStV resistance genes as well as the analysis of the resistance to the transmission of the viruses by the insect vectors.

Selected publications

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Pernet A., Hoisington D., Franco J., Isnard M., Jewell D., Jiang C., Khairallah M., Marchand J.L., Reynaud B., Glaszmann J.C., González de León D. Genetic mapping of maize streak virus resistance in the tropical 'Revolution' source. 1. Resistance in line D211 and stability against different virus clones. Theor. Appl. Genet. (submitted).

Pernet A., Hoisington D., Dintinger J., Jewell D., Jiang C., Khairallah M., Letourmy P., Marchand J.L., Glaszmann J.C., González de León D. Genetic mapping of maize streak virus resistance in the tropical 'Revolution' source. 2. Resistance in line CIRAD390 and stability across germplasm. Theor. Appl. Genet. (submitted).

Oil palm

The cultivated oil palm tree belongs to the species *Elaeis guineensis* from tropical Africa. It is allogamous and propagated via seeds. A related species from South America, *E. oleifera,* is a promising potential source of valuable characters, including reduced growth habit and disease tolerance.

Molecular analysis of genetic diversity

Molecular markers such as RFLPs and AFLPs were used to assess genetic diversity, its organization and the genetic relationship within and between *E. oleifera* germplasm of diverse origins. Analyses were performed on a set of 241 accessions of *E. oleifera* representing the geographical area of distribution for this species (Brazil, French Guyana, Surinam, Peru, Colombia, Panama, Costa Rica and Nicaragua). The results show a strong structure related to the geographical origin. A total of four distinct genetic groups were displayed: the Brazilian group; French Guyana and Surinam accessions forming one group; Peru forming another group and Colombia, Panama, Costa Rica and Nicaragua forming a single group. While the Brazilian group presented a high level of diversity (Nei's genetic diversity index = 0.245), with an intra-regional structure in accordance with the fluvial pattern of the Amazon region, no genetic diversity was detected within the other groups.

Nei's unbiased genetic distances between the different pairs of groups were variable, ranging from 0.057 between French Guyana and Surinam to 1.122 between the groups from French Guyana and from Brazil.

The genetic diversity revealed by the AFLP markers are highly in accordance with the RFLP results.

SELECTED PUBLICATIONS

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Genome mapping and tagging of useful genes

The composition of the fruit is a major component determining oil yield in *Elaeis guineensis.* It depends primarily on the thickness of the shell, which is determined by a Mendelian factor, the *Sh* gene, and by several minor genes with quantitative effects. Bulk segregant analysis (BSA) was applied to identifying AFLP markers linked to the *Sh* gene. One AFLP marker of the Sh^+ allele coding for a thick shell was identified. Such AFLP markers form a first basis for the mapping and further tagging of genes involved in fruit morphology and fertility.

Pineapple

The CIRAD collection is one of the main pineapple germplasm collections in the world. It was first gathered in West Africa and later transferred to the French West Indies (Martinique). This collection has been enriched with clones from collecting trips to Venezuela, in collaboration with the Central University of Venezuela (1984 to 1986), and more recently in Paraguay, Brazil and French Guyana, in collaboration with the EMBRAPA (Empresa Brasileira de Pesquisa Agropecuária, Brazil) with funding from the European Community. The development of this collection was coupled with the application of molecular techniques for the evaluation of the corresponding genetic diversity.

Molecular analysis of genetic diversity

A preliminary RFLP study had shown a low cytoplasmic genetic diversity. Only two cytoplasmic groups could be defined after sorting out 56 combinations (7 probes × 8 endonucleases) on 75 accessions, only one combination showing polymorphism. DNA polymorphism was further studied at the nuclear level, using the gene coding for the ribosomal RNA (rDNA) as probe. This preliminary study was followed by a more complete analysis of 92 representative clones of the main genetic groups or varieties of pineapple. These clones are part of the CIRAD collection. Eight restriction endonucleases were used in this study. Six groups could be identified, highlighting genus *Ananas* as being very homogeneous on the basis of rDNA analysis.

A global evaluation of germplasm diversity is presently under progress, including agro-morphological as well as molecular aspects, funded by an European Community cooperative project, involving CIRAD, EMBRAPA (Brazil), FONAIAP (Fondo Nacional de Investigaciones Agropecuarias, Venezuela) and University of Algarve (Portugal). The first step of the molecular evaluation is the construction of a pineapple genomic library, which is in progress. So far, 1300 gDNA probes have been obtained. Five hundred have been tested, and probe sorting in combination with six endonucleases yielded 27 combinations showing polymorphic oligogenic patterns. Ten microsatellites have been identified and are being sequenced.

RFLP probes, as well as microsatellites, will be used to study genetic diversity in a sample of 300 clones, mainly derived from recent prospections.

Probe sorting will go on in collaboration with the University of Algarve, in order to quickly broaden our set of RFLP markers, which will be used for gene mapping in Algarve.

Selected publications

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Duval M.F., Coppens d'Eeckenbrugge G., Ferreira F.R., Cabral J.R.S., Bianchetti L.B., 1997. First results from joint EMBRAPA-CIRAD *Ananas* germplasm collecting in Brazil and French Guyana. Acta Hort. 425:137-144.

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Current specific research contract

Evaluation and utilization of pineapple genetic resources from the Amazon to breed resistant varieties. European Commission-Inco n° 95-1051.

Rice

Due to its interest in rice varietal improvement and its involvement in genome analysis for several monocots, CIRAD has promoted the analysis of the genome of rice, both as a crop and as a model. This has been done largely in collaboration with ORSTOM and IRRI (International Rice Research Institute, the Philippines).

Genetic resources and tagging of useful genes

The research on rice as a crop concerns the organization of the genetic resources and the localization of genes of agricultural interest.

Numerous studies on molecular diversity in the genus *Oryza* have been conducted at ORSTOM. CIRAD has focussed on the species *O. sativa.* The most recent studies involve the development and the characterization of a small nucleus collection. CIRAD is also coordinating a European project on Mediterranean rice genetic resources and using microsatellite markers to investigate genetic diversity.

Linkage analyses are directed towards the mapping of genes controlling various traits, such as grain aroma, resistance to the rice blast fungus, resistance to the rice yellow mottle virus, plant architecture or root attributes.

Rice genome, a model

The use of rice as a model genome is the focus of the latest developments. In collaboration with ORSTOM, CIRAD is partner in several European projects, such as in the Biotech frame:

– Egram (European Gramineae mapping initiative), which aims at developing various tools on rice (populations, DNA libraries) for facilitating the access, through comparative mapping, to useful genes in European cereals and forages, in particular disease resistance genes; CIRAD and ORSTOM produce recombinant inbred (doubled haploid or single seed descent) lines from the cross IR64 × Azucena and build a reference map for anchoring regions of interest in other plants through mapping of heterologous probes;

– Rice transposon mutagenesis, which aims at creating a wide range of insertion mutants for subsequent gene cloning, by transformation with AC/DS transposable elements; CIRAD, in association with ORSTOM, participates in the genetic transformation and the screening of mutants for resistance to blast.

The rice resources thus developed will be complemented by a rice BAC library with the support of INRA.

Selected publications

Courtois B., Huang N., Guiderdoni E., 1995. RFLP mapping of genes controlling yield components and plant height in an indica/japonica doubled-haploid population of rice. In: Fragile lives in fragile ecosystems. Los Banos: IRRI, p. 963-976.

Glaszmann J.C., Mew T., Hibino H., Kim C.K., Vergel de Dios-Mew T.I., Vera Cruz C.M., Notteghem J.L., Bonman M.J., 1995. Molecular variation as a diverse source of disease resistance in cultivated rice. In: Rice genetics III. 1995 Oct. 15-20. Los Banos: IRRI, p. 460-465.

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Yadav R., Courtois B., Huang N., McLaren G., 1997. Mapping genes controlling root morphology and root distribution in a doubled-haploid population of rice. Theor. Appl. Genet. 94:619-632.

Current specific research contracts

Rice genetic resources for Europe. European Union, Genres, 1996-1999.

European Gramineae mapping initiative. European Union, Biotech, 1997-2000.

Rice transposon mutagenesis. European Union, Biotech, 1997-2000.

Constitution d'une banque BAC chez le riz en appui au marquage et au clonage de gènes chez les graminées. AIP Génome, INRA, 1997-1998.

Rubber tree

The worldwide natural rubber production is based on the cultivation of one species only: *Hevea brasiliensis* (Euphorbiaceae, 2n = 36). This tree species, native to the Amazon basin, was introduced into the Old World at the end of the XIXth century. All the clones cultivated today, called Wickham clones, come from a few seeds collected in 1876 by H. Wickham in a small location near the Tapajos River in Brazil.

More recently, international surveys were carried out in the Amazon basin in order to broaden the genetic base of the domesticated material. Nevertheless, the use and the management of this germplasm was rendered difficult due to the lack of knowledge on the genetic organization of rubber tree. CIRAD researchers obtained original results on genetic diversity and genome organization of this species by developing a molecular marker approach.

Molecular analysis of genetic diversity

Genetic diversity of *H. brasiliensis* was analyzed using both isozymes and RFLPs (cytoplasmic and nuclear) on collections of accessions (clones), including Wickham and wild Amazonian clones originating from Peru, Columbia and three states of Brazil (Acre, Rondonia, Mato Grosso).

Between 8 and 14 isozyme loci were characterized on a sample of 800 clones. Nuclear RFLPs were applied on a subset of 162 Brazilian and Wickham clones using 25 single copy homologous probes and one rDNA probe revealing two loci. A high level of genetic diversity was observed. Mitochondrial RFLPs, analyzed in a sample of 395 clones, revealed a high level of genetic diversity among Amazonian populations, whereas chloroplastic DNA appeared little polymorphic. Both cytoplasmic components were nearly monomorphic in the cultivated population.

In contrast to agro-morphological traits, molecular markers revealed a great extent of genetic diversity and a clear genetic structurating of Amazonian populations in accordance with geographic origin.

The results obtained with the different types of molecular markers are similar (Figure 1) and lead to the following conclusions:
– six genetic groups can be distinguished in *Hevea* germplasm;
– each group encompasses populations of close geographic origins;
– gene flows and consequently genetic relationships between populations follow the patterns of the Amazon River network;
– comparatively, genetic diversity of the cultivated clones appears reduced, mainly at the cytoplasmic level.

Differences observed between isozyme and nuclear RFLP seem to be due to sampling effects either for accessions or for genetic markers used.

Current research is directed towards the following aspects: 1) effect of the sampling of mapped molecular markers, 2) relationships between agro-morphological variation and molecular diversity, and 3) strategies for building a core collection.

Particular efforts aim at the technical transfer and routine application of molecular markers on breeding sites. Isozymes are convenient markers; a database has been constituted, that contains the fingerprints of 300 selected clones. A mobile electrophoresis kit has been designed and utilized to carry out conformity checks directly in plantations. Since its design, the electrophoresis kit and its operator have carried out assessments for research center or estate plantations in 9 different countries. In addition, the technique has been transferred in Brazil, Cambodia, Côte d'Ivoire, Guatemala, Mexico and Vietnam. The development of SSR markers in rubber tree is also pursued with the objective to provide simple routine techniques, in complement to isozymes, for actual integration of molecular markers into breeding programs.

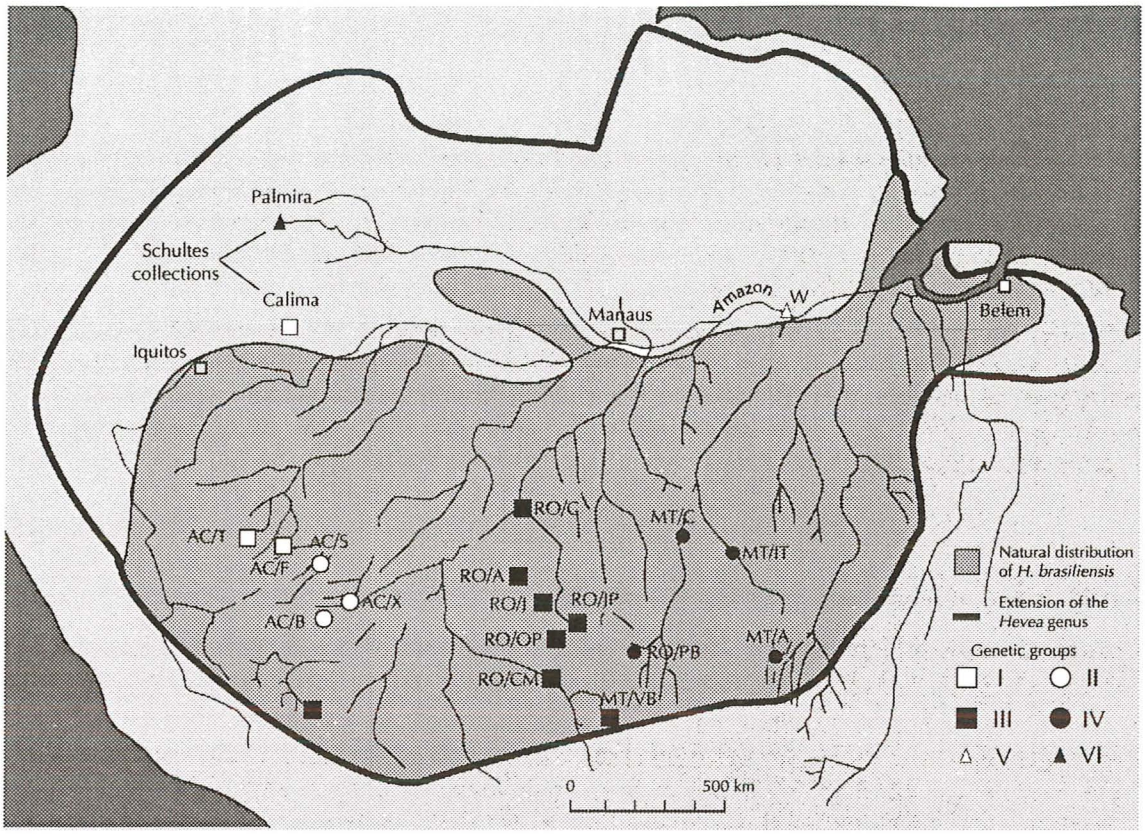

Figure 1. Correspondence between geographic origin and genetic relationship in H. brasiliensis *germplasm. Each symbol corresponds to one of the 6 genetic groups (I to VI) identified at the molecular level (isozymes and RFLP). Exact collect location is unknown for the accessions collected in Columbia (Schultes collections), and belonging to the genetic groups I and VI. AC: Acre; RO: Rondonia; MT: Mato Grosso; W: Wickham (cultivated clones).*

Selected publications

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Hamon S., Dussert S., Chantereau J., Chevallier M.H., Deu M., 1998. Effects of quantitative and qualitative Principal Component Score strategies on the structure of coffee, rice, rubber tree and sorghum core collections. Génét. Sél. Evol. (in press).

Current specific research contract

Essai d'optimisation du choix des individus et des descripteurs (moléculaires et agro-morphologiques) pour une meilleure utilisation des collections de ressources génétiques. Projet ORSTOM-CIRAD, Bureau des Ressources Génétiques, 1997-2000.

Genome mapping and tagging of useful genes

H. brasiliensis, as the nine other *Hevea* species, was considered as a tetraploid with 2n = 4x = 36 chromosomes. Genetic map construction using RFLP markers revealed a diploid organization of the genome, with rare duplicated loci, in disaccordance with cytogenetic observations. Nevertheless, the mapping results were validated through the segregation and linkage analyses of 300 specific markers (isozymes, RFLP and SSR) in two different progenies.

The first progeny, obtained by selfing of the cultivated clone PB260, encompasses 75 F_2 offsprings. The map was built using mainly 160 RFLP markers complemented with 5 isozyme, 30 RAPD and 14 SSR markers. This population was chosen for further QTL analyses of latex yield and growth vigor.

The second progeny is an F_1 cross between 2 heterozygous clones: PB260 as female and RO38, an Amazonian clone from Brazil, as male. The segregation data on 109 offsprings were scored considering this population as a double pseudo-testcross. The map encompasses 285 loci, mainly RFLPs (249 markers) with in addition 8 isozymes, 2 rDNA loci and 26 SSR. On this population, QTL analysis is currently being performed for resistance to the South American leaf blight (SALB) disease caused by *Microcyclus ulei* (Ascomycete). This endemic disease has severely limited *Hevea* monoculture in Latin America and threatens plantations located in Asia.

The synthetic map from the 2 progenies encompasses 332 markers located in 22 independent linkage groups and 12 unlinked markers (Figure 2). To our knowledge, this is the first map established for this species.

Recent application of AFLP markers led to the identification of 300 additional markers which permitted saturation of the *Hevea* genetic map and determination of the 18 basic linkage groups.

The 30 mapped SSR markers so far available were identified from a homologous genomic library constructed in the laboratory. Usefulness of SSR markers will be enhanced by their adaptation to technological transfer and overseas application.

Selected publications

Seguin M., Besse P., Lespinasse D., Lebrun P., Rodier-Goud M., 1996. *Hevea* molecular genetics. Plant. Rech. Dév. 3:77-88.

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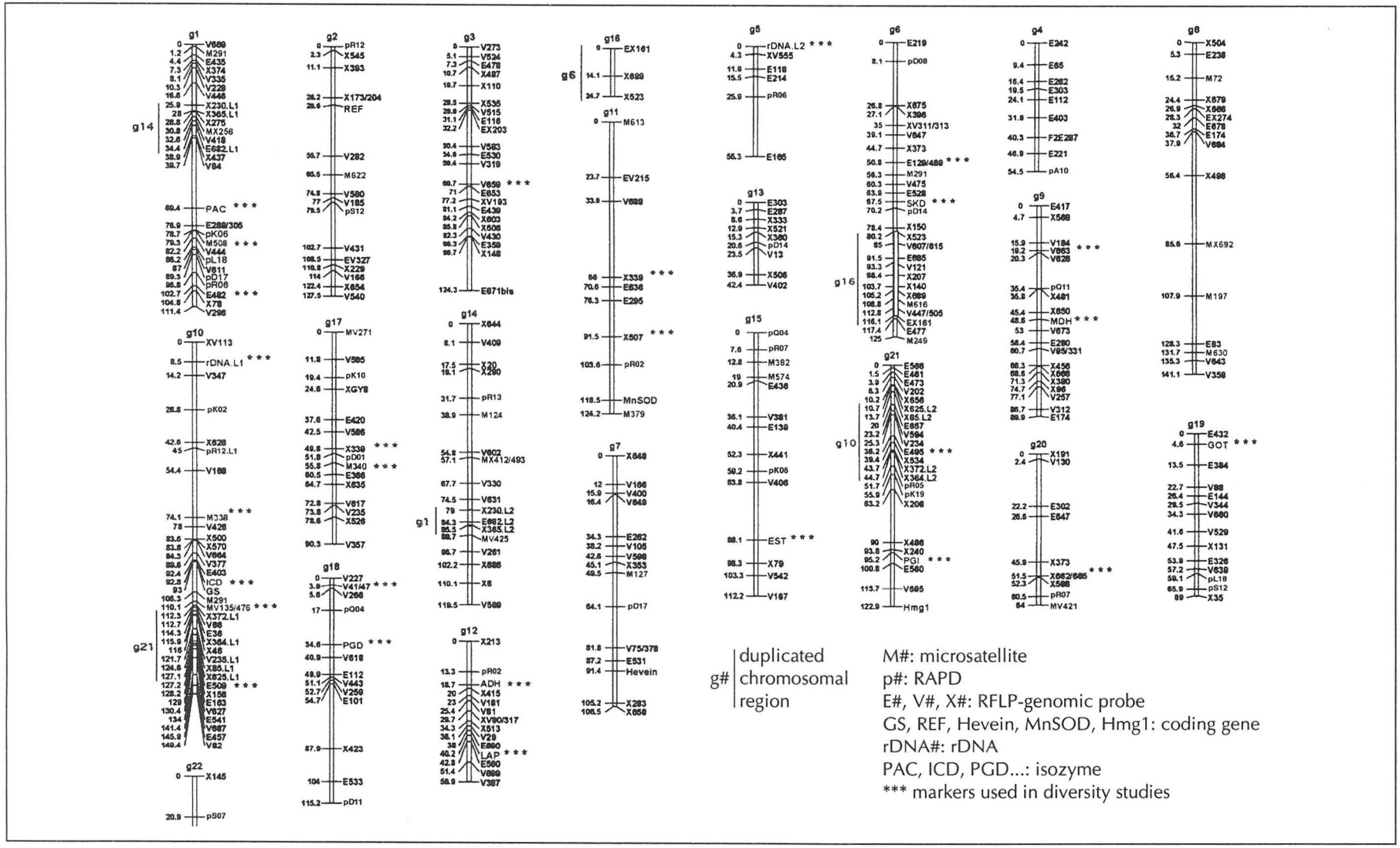

Figure 2. Synthetic map of Hevea.

Sorghum

Sorghum is a staple food crop in Africa. CIRAD has conducted breeding experiments for selecting varieties adapted to West Africa. Most locally adapted cultivars belong to the race guinea; molecular diversity has been used to investigate the relation of this race to the other races of sorghum. A particular expertise has been built on genetic diversity and is being used in order to build a core collection. Recent genetic analyses have been directed to improving grain quality in relation to the traditional preparation as Tô; a QTL mapping project is being conducted. Present experiments also aim at mapping genetic factors for insect resistance. Most of these efforts are in collaboration with ICRISAT (International Crops Research Institute for the Semi-Arid Tropics, Mali) and Rustica.

Molecular analysis of genetic diversity

Sorghum (*Sorghum bicolor* subsp. *bicolor*) varieties are traditionally classified into 5 main races on the basis of morphological traits, especially panicle and grain traits. Isozyme diversity provided a new insight into genetic diversity, and showed a marked geographic structure. RFLP analysis was performed on 94 varieties chosen to represent the main cross combinations (race × geographic origin), using 35 maize probes that detect polymorphism with at least one of the two restriction enzymes *Hind*III and *Xba*I. A total of 50 polymorphic probe-enzyme combinations yielded 158 polymorphic bands.

The bicolor race appeared highly variable and included many rare markers. Among the other races multivariate analysis of the data differentiated six clusters corresponding, by decreasing magnitude of divergence, to the margaritiferum types (a subrace of race guinea); the guinea forms from western Africa; race caudatum; race durra; race kafir; guinea forms from southern Africa. The apparent geographic differentiation was related to the contrasting distribution of these races and to a higher similarity between races localized in southern Africa. The data agree with the current hypotheses on sorghum domestication but reveal associations between neutral markers and traits probably highly subjected to human selection.

Wild sorghum is diploid or tetraploid and African sorghum (*S. bicolor* subsp. *verticilliflorum*) is subdivided into four races, considered to be progenitors of cultivated sorghum. Mitochondrial DNA analyses were performed to compare the diversity of wild and cultivated sorghum and study the genetic origin of guinea margaritiferum.

Our results confirmed the specificity of guinea margaritiferum and demonstrated the presence of two genetic entities within this subrace. Another guinea group was also noted, which corresponded to Asian guinea roxburghii. In wild sorghum, the arundinaceum race appeared to be homogenous while the verticilliflorum race was separated into two groups, one associated with the arundinaceum race. The diversity observed in cultivated forms was found to be encompassed within the wild-type pool, except for one guinea margaritiferum group. There did not seem to be any particular relationship between wild and cultivated races.

The data available for molecular markers and agro-morphological traits are being used in collaboration with ORSTOM in order to test the efficiency of various strategies for sampling genetic diversity.

Selected publications

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Genome mapping and tagging of useful genes

A sorghum composite linkage map was constructed with heterologous probes already mapped on maize and sugarcane, based on two recombinant inbred line populations (RIL). This map includes 199 loci revealed by 188 probes and distributed on 12 linkage groups (Figure 5). A comparison was performed between the sorghum composite map and the published map of sugarcane cultivar R570 based on the 83 probes they had in common, revealing generalized synteny and colinearity. Recent results are confirming that the basic genome maps of the two main species that contributed to modern sugarcane are differing very little from that of sorghum.

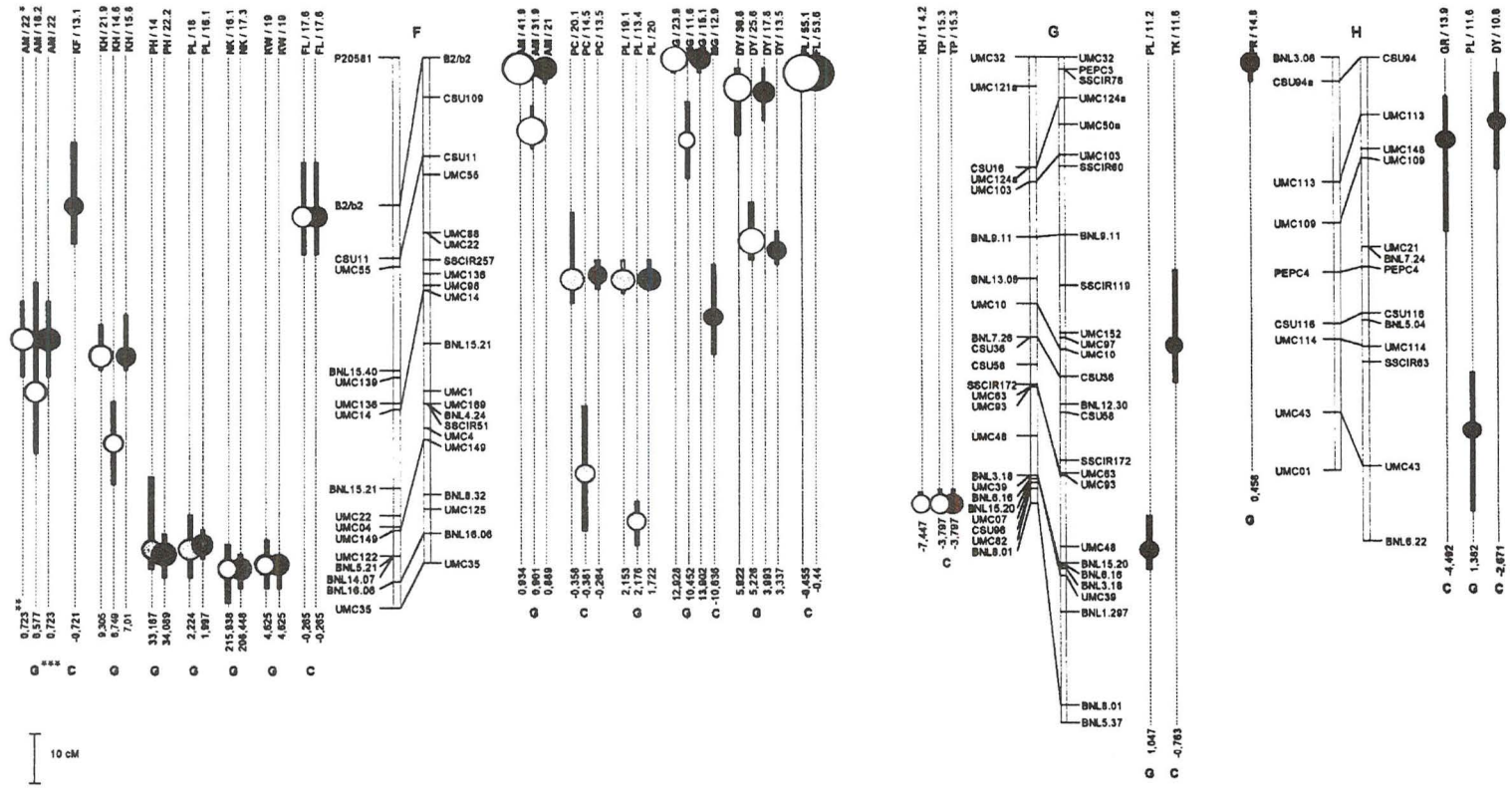
The comparison between the consensus sorghum-sugarcane map and maize map reveals that the maize genome has a complex structure. A particular example was more thoroughly described; a series of linkage blocks with conserved gene orders appeared obvious between maize chromosome 3 and 8 and homoeologous sorghum linkage groups; however, these blocks were interspersed with non-homoeologous regions corresponding to the central part of the two maize chromosomes and they revealed several inversions in maize compared to sorghum-sugarcane.

Recently, a more extended RFLP map was produced using sorghum cDNA probes and several sets of heterologous probes mostly mapped on rice. This new RFLP map includes 299 loci distributed on 12 linkage groups and two minute clusters of two or three loci, spanning a total genetic distance of 1404 cM. Comparing this map to the most advanced sorghum linkage maps made in other laboratories, two couples of large groups remain to be merged while the two separate clusters have to be assigned to complete linkage groups.

The AFLP technique was applied for further extending genome coverage. The AFLP linkage map currently consists of 137 loci distributed in 11 linkage groups covering 849 cM (31 markers remained independent). The integration of AFLPs into an existing linkage map based on RFLP markers led to an increase of the map length (1932 cM versus 1404 cM for the RFLP map); in most cases, AFLP clusters filled in regions poorly marked or corresponding to earlier gaps, leading to the merging of previously distinct clusters. The map is now close to saturation.

Linkage maps have been used to locate quantitative trait loci (QTL) for several grain traits. Phenotypic correlations between the traits studied are generally in accordance with the corresponding QTL location. In particular, the results indicate that panicle traits, grain productivity and germinative ability are controlled by linked genes or genes with pleiotropic effects. Interestingly, major QTLs for amylose content dehulling yield and kernel flouryness are not linked to productivity traits, while they are co-located with major QTLs for molds during germination and the *B2/b2* gene (testa layer).

Head bug resistance gene mapping is just starting. The molecular map will be conducted on 250 F_2 progenies derived from a cross between a caudatum genotype (source of resistance, Malisor 84-7) and a caudatum improved line (source of susceptibility, S34).



* Trait / Percentage of phenotypic variance explained by the QTL
Traits: PH (plant height), PL (panicle length), PC (panicle compactness), NK (number of kernel/panicle), TK (1000 kernel weight), KW (kernel weight/panicle), TP (threshing percent), DY (dehulling yield), FL (kernel flouryness), KF (kernel friability), KH (kernel hardness), AM (amylose content), PR (protein content), IL (lipid content), GR (germination rate), MG (molds during germination), MR (molds after harvest)
** Additivity effect.
*** Racial origin of the positive QTL effect (G: guinea, C: caudatum)

Figure 5. Genetic maps of sorghum and QTLs detected for population RIL 249 (left) and RIL 379 (right). Each QTL is represented with a circle located on the LOD peak and with a box representing confidence interval. White QTLs were detected with SIM; black QTLs were detected with CIM.

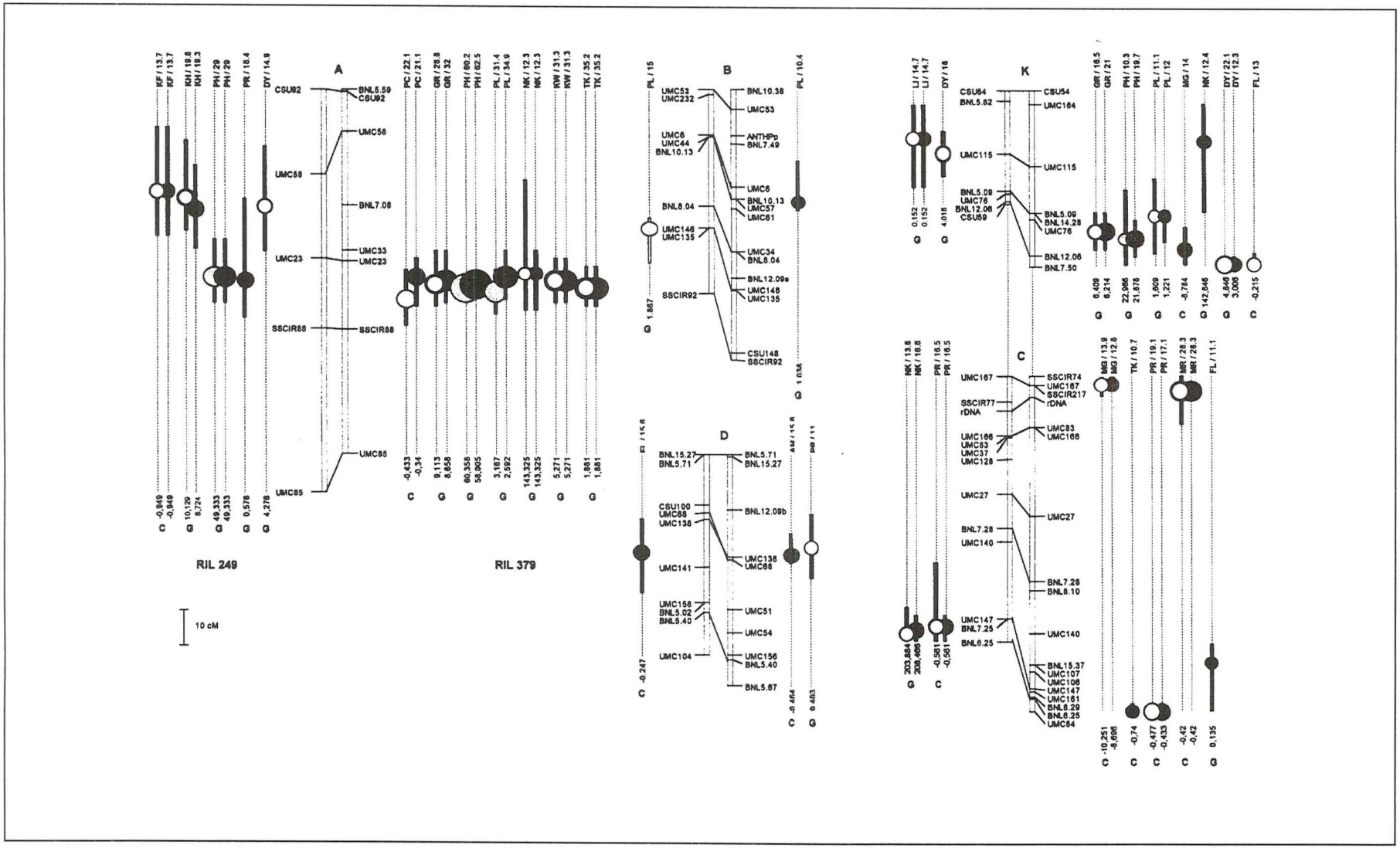


Figure 5. Continued

Selected publications

Dufour P., Grivet L., D'Hont A., Deu M., Trouche G., Glaszmann J.C., Hamon P., 1996. Comparative genetic mapping between duplicated segments on maize chromosomes 3 and 8 and homoeologous regions in sorghum and sugarcane. Theor. Appl. Genet. 92:1024-1030.

Dufour P., Deu M., Grivet L., D'Hont A., Paulet F., Bouet A., Lanaud C., Glaszmann J.C., Hamon P., 1997. Construction of a composite sorghum genome map and comparison with sugarcane related complex polyploid. Theor. Appl. Genet. 94:409-418.

Glaszmann J.C., Dufour P., Grivet L., D'Hont A., Deu M., Paulet F., Hamon P., 1997. Comparative genome analysis between several tropical grasses. Euphytica 96:13-21.

Boivin K., Deu M., Rami J.F., Trouche G., Hamont P., 1998. Towards a saturated sorghum map using RFLP and AFLP markers. Theor. Appl. Genet. (in press).

Rami J.F., Dufour P., Trouche G., Fliedel G., Mestres C., Davrieux F., Blanchard P., Hamon P., 1998. Quantitative trait loci for grain quality, productivity, morphological and agronomical traits in sorghum (*Sorghum bicolor* L. Moench). Theor. Appl. Genet. (in press).

Current specific research contracts

Cartographie génétique de facteurs de qualité du grain chez le sorgho et le maïs. Rustica-Prograin Génétique, bourse Cifre, 1996-1998.

Amélioration durable de la production de sorgho en Afrique de l'Ouest par la lutte intégrée contre ses insectes ravageurs. European Union, DG XII IC 18-CT96-0106, 1996-1999.

Sugarcane

Sugarcane is a vegetatively propagated crop with a complex genome. The genus *Saccharum* is characterized by a high ploidy level with chromosome numbers ranging from 40 to above 140. The *S. officinarum* clones initially cultivated have been replaced in the 1920s by materials introgressed by wild species, mainly *S. spontaneum.* Since then, most cultivated sugarcane varieties have kept this hybrid and aneuploid genomic structure: approximately a hundred *S. officinarum* chromosomes associated with a few *S. spontaneum* chromosomes.

Several research groups in the USA are involved in sugarcane genome analysis using the ancestral species. Researchers at CIRAD developed a specific approach inspired by early isozyme analyses and tried to characterize the interspecific aneuploid genome structure of modern cultivars.

Molecular analysis of genetic diversity

Genetic diversity revealed with RFLPs was analyzed in a sample of 50 clones representing various taxonomic groups of the *Saccharum* complex using heterologous probes, 2 of which were chloroplastic, 9 were mitochondrial, 1 represented a repeated nuclear sequence covering ribosomal DNA and 33 represented low copy nuclear sequences. The results confirmed previous taxonomic schemes derived from morphological, cytological and biochemical data, and brought about novel information:

– mitochondrial diversity was observed between and within *S. spontaneum* and *S. robustum;* diversity is larger in *S. spontaneum; S. robustum* displays two types, one of which characterizes all *S. officinarum* clones; this allows excluding part of *S. robustum* clones from potential ancestors of *S. officinarum;*

– rDNA diversity showed i) an opposition between the Indian forms and the large chromosome numbered southern forms within *S. spontaneum,* ii) the presence in low dosage of markers of the southern *S. spontaneum* forms in *S. officinarum;*

– diversity revealed with low copy nuclear sequences showed the same geographic structure within *S. spontaneum.*

RFLP with nuclear probes is being extensively applied to commercial varieties. Besides providing an exceedingly powerful identification tool, these probes allow characterizing the fraction of the *S. spontaneum* genome still present in modern varieties and analyzing the diversity in terms of respective contribution of the ancestral species and trends towards specific adaptation to contrasting environments.

These data will help broaden the genetic base of modern varieties and foster the exploitation of the diversity available in germplasm collections.

Recent progress in molecular cytogenetics has enabled to determine the basic chromosome numbers in the ancestral species that contributed to modern sugarcane cultivars and to study genus-specific repetitive sequences within the *Saccharum* complex.

Selected publications

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Lu Y.H., D'Hont A., Paulet F., Grivet L., Arnaud M., Glaszmann J.C., 1994. Molecular diversity and genome structure in modern sugarcane varieties. Euphytica 78:217-226.

Paulet F., Glaszmann J.C., 1994. Les biotechnologies en soutien à la diffusion variétale chez la canne à sucre. Agric. Dév. 2:55-61.

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Alix K., Baurens F.C., Glaszmann J.C., D'Hont A., 1998. Isolation and characterization of a satellite DNA family in the *Saccharum* complex. Genome (in press).

D'Hont A., Ison D., Alix K., Roux C., Glaszmann J.C., 1998. Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of RNA genes. Genome (in press).

CURRENT SPECIFIC RESEARCH CONTRACT

Investigation of the *S. spontaneum* contribution to commercial clones by genomic DNA *in situ* hybridization. Sugar Research and Development Corporation, Australia, 1996-1999.

Genome mapping and tagging of useful genes

Modern sugarcane varieties are complex aneuploids and typically have chromosome numbers in the 100-125 range with about 5-10% of them contributed by wild relatives, mainly *S. spontaneum* and the rest by *S. officinarum.* This particular genomic constitution is favorable for mapping the *S. spontaneum* genome because there are few chromosomes involved; there is a marked polymorphism between *S. officinarum* and *S. spontaneum,* so that markers borne by *S. spontaneum* chromosomes are easily distinguishable. We conducted the RFLP analysis of individuals derived from selfing of modern varieties using maize and sugarcane DNA probes.

A vast study is going on with the self progeny of variety R570, including cosegregation analysis between molecular markers and agronomic traits, in order to explore the prospects for marker-assisted selection. More than 600 markers have been located onto some 100 cosegregation groups, forming 10 linkage groups. At least 25 chromosome portions derived from *S. spontaneum* have thus been marked. Recent progress in *in situ* hybridization now enables differential labeling of this fraction on chromosome spreads (Photographs 1 to 3). Significant genetic factors have been identified, which contribute to rust resistance and yield components. The study is being expanded to a larger population. The use of AFLPs has recently improved the quality of the map by more than 700 new markers.

The genome colinearity with maize and sorghum has been clearly established; it will allow transposing information obtained from simpler crops and applying it to sugarcane (Figure 6).

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Grivet L., D'Hont A., Dufour P., Hamon P., Roques D., Glaszmann J.C., 1994. Comparative mapping of sugarcane with other species within the tribe Andropogoneae. Heredity 73:500-508.

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D'Hont A., Grivet L., Feldmann P., Rao P.S., Berding N., Glaszmann J.C., 1996. Characterization of the double genomic structure of modern sugarcane cultivars, *Saccharum* spp., by molecular cytogenetics. Mol. Gen. Genet. 250:405-413.

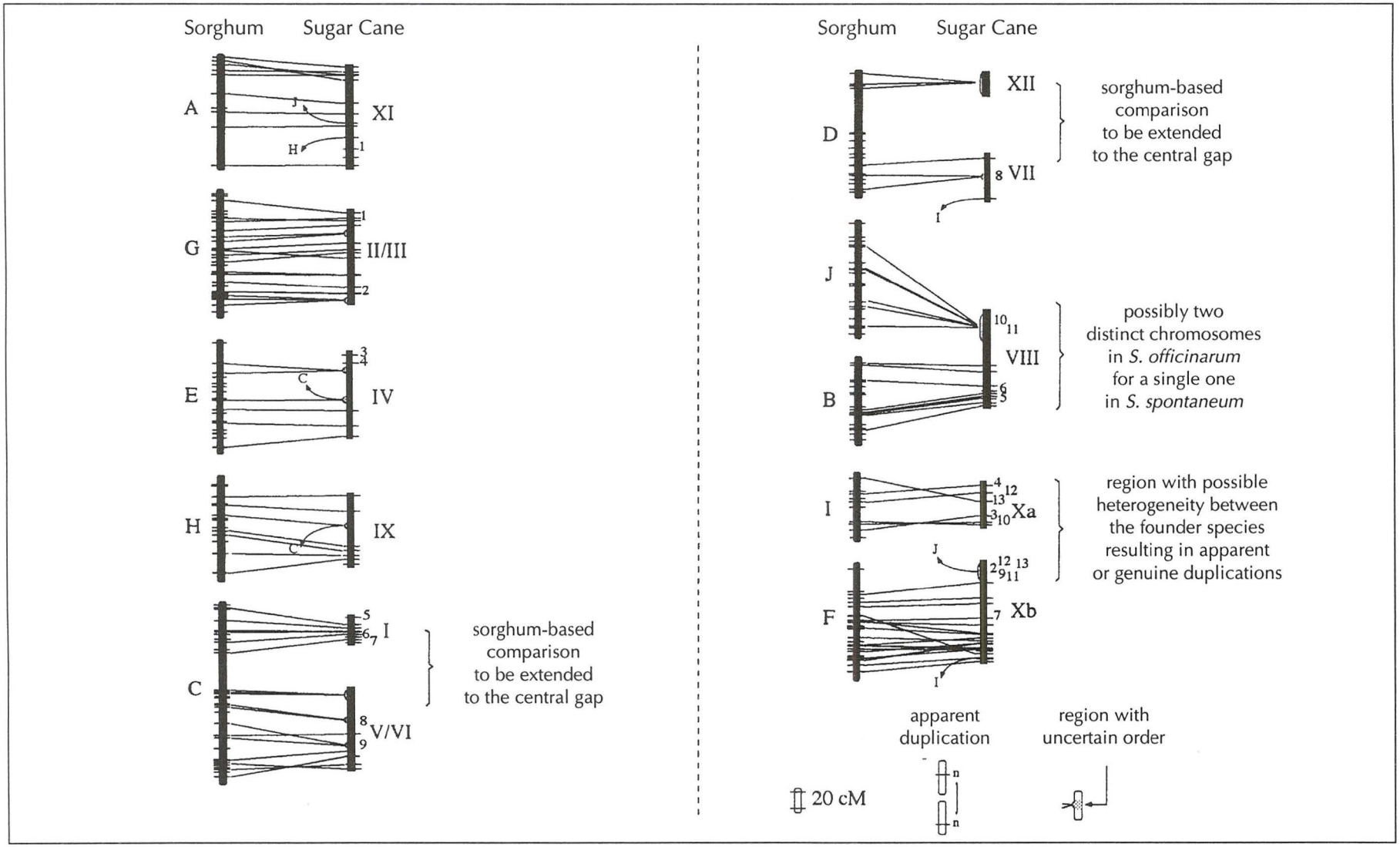

Figure 6. Colinearity between the genetic maps of sugarcane and sorghum.

Dufour P., Grivet L., D'Hont A., Deu M., Trouche G., Glaszmann J.C., Hamon P., 1996. Comparative genetic mapping between duplicated segments on maize chromosomes 3 and 8 and homoeologous regions in sorghum and sugarcane. Theor. Appl. Genet. 92:1024-1030.

Grivet L., D'Hont A., Roques D., Feldmann P., Lanaud C., Glaszmann J.C., 1996. RFLP mapping in cultivated sugarcane (*Saccharum* spp.): genome organization in a highly polyploid and aneuploid interspecific hybrid. Genetics 142(3):987-1000.

Dufour P., Deu M., Grivet L., D'Hont A., Paulet F., Bouet A., Lanaud C., Glaszmann J.C., Hamon P., 1997. Construction of a composite sorghum genome map and comparison with sugarcane, a related complex polyploid. Theor. Appl. Genet. 94:409-418.

Glaszmann J.C., Dufour P., Grivet L., D'Hont A., Deu M., Paulet F., Hamon P., 1997. Comparative genome analysis between several tropical grasses. Euphytica 96:13-21.

CURRENT SPECIFIC RESEARCH CONTRACT

Fine mapping of a rust resistance gene in sugarcane cultivar R570 in view to its positional cloning. International Consortium for Sugarcane Biotechnology, 1997-1999

Somatic embryogenesis and genetic transformation

Banana and plantain

Cultivated bananas and plantains being parthenocarpic fruits, subsequent crops are usually produced from suckers of the mother plant. The use of vitroplants obtained by *in vitro* budding is one of the main improvements in the cultivation of banana during the last decade. Among crops cultivated on a large scale, banana is the most widely *in vitro* propagated plant (the worldwide production amounts to about 50 million plants/year). In addition to the advantages of using little space and providing continuous availability, the plants produced by this technique have a very high sanitary quality. If combined with appropriate agronomic practices, the vitroplants may produce high-yielding crops while reducing pesticide use (nematicides). However, the cost of the vitroplant produced by *in vitro* budding remains high compared to conventional methods. This high cost is the most significant obstacle to its common use. Somatic embryogenesis is likely to significantly reduce such a cost (reduction of labor cost). In the near future, it is planned to apply the technique to traditional cultivars but also to new hybrids from genetic improvement programs in order to satisfy the probable high demand for varietal reconversion. The development of an embryogenesis technique is also of interest for the development of genetic transformation and somatic hybridization.

Somatic embryogenesis for mass micropropagation

Investigations in various laboratories have targeted three main methods of somatic embryogenesis in relation to three different explant types: the bases of leaf sheaths or rhizome fragments of plants produced *in vitro;* thin sections of highly proliferating bud cultures placed in liquid medium; young male flowers. For over 10 years, CIRAD has been working on somatic embryogenesis starting form young male flowers. This latter material seems to date to be the technique with the best results when applied to a large number of cultivars, including the Cavendish group which is the most commonly cultivated (Photographs 17 to 22).

CIRAD masters the whole system from the initiation of embryogenic tissue to the regeneration of plantlets through a cell suspension phase. Friable white calluses with numerous proembryos are obtained from young male flowers cultivated for 5 to 6 months on a solid medium. After a transfer into a liquid medium, cell suspensions are initiated and can be maintained during more than 15 months or cryopreserved in liquid nitrogen. Plating of 1 ml of packed cells from suspension leads to the formation of more than 10^5 embryos of which 5-40% can germinate according to the cultivars (AA, AAA and AAB genotypes). Histological analysis underlines the unicellular origin of the embryos meaning that cell suspensions can be used as targets for genetic transformation.

Positive results have been recently obtained jointly by CIRAD and CATIE regarding the application of the embryogenesis technique to banana plants without male flowers by the use of female ones.

A significant part of the research currently conducted focuses on the study of the agronomic conformity of the regenerated plants in the field. On the traditional cultivar of the sweet banana Grand Nain, plants originated from somatic embryogenesis are true-to-type, with a fruit yield similar to plants derived from *in vitro* budding. CIRAD expertise is also strengthened by a 10-year period of experience in commercial production of banana vitroplants through its subsidiary Vitropic. Technical agronomic practices particularly suitable to vitroplants have been developed. A feasibility program for banana mass production through somatic embryogenesis is going on with Vitropic. The main objective is to adapt the technique to the requirements of commercial production (changes in scale production, reduction in costs, quality control).

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Escalant J.V., Teisson C., Côte F.X., 1994. Amplified somatic embryogenesis from male flowers of triploid banana and plantain cultivars (*Musa* sp.). In Vitro Cell. Dev. Biol. Plant 30:181-186.

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Grapin A., Schwendiman J., Teisson C., 1996. Somatic embryogenesis in bananas and plantains. In Vitro Cell. Dev. Biol. Plant 32:66.

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CURRENT SPECIFIC RESEARCH CONTRACTS

Optimization of new strategies for genetic improvement in bananas for local market. European Union, Inco-DG XII 18CT970204, 1998-2001.

Somatic embryogenesis for mass propagation in banana: study of the scale-up. ANVAR (French national agency for research valorization), 1997-1999.

Genetic transformation

Bananas and plantains are seriously threatened by pests and numerous viral and fungal diseases. Thus resistance to biotic stresses is an important part of regional or national efforts. Genetic transformation is of interest because (i) cultivated bananas are triploid and sterile (ii) some resistance sources are not available among genetic resources (i.e. virus resistance).

Genetic transformation of banana is recent. Stable transformation has been obtained using meristems through *Agrobacterium* or cell suspensions through particle bombardment. But the recent Pro*Musa* congress organized jointly by INIBAP (International Network for the Improvement of Banana and Plantain) and the World Bank concluded on the need to improve transformation techniques in order to integrate them rapidly into the improvement programs. The main requirements were (i) a more efficient pre-transformation tissue culture system through somatic embryogenesis, (ii) the improvement of genetic transfer methods including the development of a molecular tool box to control gene expression, (iii) the identification of promoters including those from banana. Different laboratories are working on these topics and are part of the BIP (banana improvement program) framework executed by the World Bank and supported by the Common Funds for Commodity (CFC).

The genetic transformation work at CIRAD is integrated into a general *Musa* improvement program. The specific expertise of CIRAD in genetic transformation originates from its good mastery of cellular regeneration systems on banana and plantain main groups and from its research on *Musa* germplasm characterization and genome mapping. This knowledge will be of particular interest for molecular genetic work aimed at gene mapping and cloning.

Researchers at CIRAD developed a gene transfer method using embryogenic cell suspensions from male flower cultures as cellular target and particle bombardment as DNA transfer method. Embryogenic cell suspension regeneration was obtained on several clones belonging to AA, AAA and AAB genotypes. Histological analysis underlined the unicellular origin of the embryos. Six plasmids containing the β-glucuronidase gene driven by different promoters were tested. The highest expressions were obtained with the CaMV35S and Ubi-Ubi exon-Ubi intron promoters. Biolistic methods with the Biorad PDS-1000/He device was used for genetic transformation. A mean higher than 5000 blue loci per shooting was regularly obtained when embryogenic cell suspensions were bombarded with 1 Um gold particles coated with the pACH25 (*bar* and *gus*

genes driven by the same pUGC1 promoter) plasmid under a pressure of 1550 PSI and a distance of 9 cm. Using the previous physical conditions, French Sombre cell aggregates were shot with the pILTAB plasmid (*gus* and *hph* genes driven by the CaMV35S promoter). Four days after shooting, the selected agent (hygromycine, 50 mg/l) was added to the culture medium. This selective agent was maintained during the whole regeneration process. Embryo development and subsequent plantlet regenerations were obtained. Transgene integration was confirmed through southern blot analysis. This protocol was successfully repeated on different suspension batches.

Research will be now focused on the improvement of the method reliability on different cultivars and the actual integration of genetic transformation methods to the improvement programs through gene cloning and transformation of improved diploid varieties used for triploid hybrids synthesis.

Selected publications

Grapin A., 1995. Régénération par embryogenèse somatique en milieu liquide et transformation génétique par biolistique de bananiers di et triploïdes. Thèse de doctorat. Montpellier: ENSAM, 90 p.

Côte F.X. Domergue R., Monmarson S., Schwendiman J., Teisson C., Escalant J.V., 1996. Embryogenic cell suspensions from the male flower of *Musa* AAA cv. Grand Nain. Physiol. Plant. 97:285-290.

Grapin A., Schwendiman J., Teisson C., 1996. Somatic embryogenesis in bananas and plantains. In Vitro Cell. Dev. Biol. Plant 32:66.

Côte F.X., Legavre T., Grapin A., Valentin B., Frigout O., Babeau J., Meynard D., Bakry F., Teisson C., 1997. Genetic transformation of embryogenic cell suspension in plantain (*Musa* AAB) using particle bombardment. In: Proceedings of the international symposium on biotechnology of tropical and subtropical species: Part 1. Acta Hort. 460.

Analysis of somaclonal variation

In banana, somaclonal variation generally affects shoots, with giant, dwarf or variegated phenotypes for the most important variant types. To date, somaclonal variation affecting *in vitro* propagated banana is not well understood, suggesting a complex genetic cause of this phenomenon. Several factors could explain or contribute to the appearance of variants: strictly genetic alteration of the plant genome as well as epigenetic modification.

A molecular biology-based approach of somaclonal variation analysis in banana was initiated. Two different and complementary approaches are being developed:
– a "shot gun" method using the AFLP technique which is able to produce an important number of genetic markers in a reasonable amount of time. The linkage of these markers to the dwarf variation will be investigated;
– a more physiological approach involving the expression of genes revealed by differential display. This approach is complemented with the study of the gibberellin biosynthesis pathway genes as gibberellins are known to be involved in processes causing dwarfism in plants.

Current specific research contract

Obtention de marqueurs moléculaires pour l'identification de variants nains de vitroplants de bananier. Vitropic, French Ministry of Research, DRRT-Languedoc-Roussillon.

Citrus

Protoplast fusion is an important component of new citrus breeding programs. Indeed it enables to overcome some constraints associated with sexual hybridization and genetic structure of citrus cultivars. Somatic hybridization can be used in rootstock improvement programs to accumulate genes of tolerance to biotic and abiotic factors. This application of somatic hybridization has been widely explored by research groups in Florida and Japan. Protoplast fusion also shows promise for the development of triploid cultivars. Indeed the pool of tetraploid parents used for sexual hybridization with diploid plants could be enriched considerably with new highly heterozygous allotetraploid hybrids. Moreover, somatic hybridization between diploid and haploid genotypes, that was first developed at CIRAD, opens very promising ways for triploid cultivar breeding. This new method of triploid synthesis enables to add a haploid genome to selected diploid cultivars without recombination.

Somatic hybridization

CIRAD started in 1994 a program of somatic hybridization in citrus in order i) to create new rootstocks associating complementary traits of tolerance to biotic and abiotic factors particularly critical for the Mediterranean basin, ii) to diversify the pool of tetraploid parents for further triploid easy peelers breeding, iii) to make direct synthesis of triploid by haploid + diploid protoplast fusion.

An efficient protocol has been developed, enabling the regeneration of plantlets 4 months after protoplast fusion. Somatic hybridization is done by electrofusion of protoplasts isolated from embryogenic callus lines for at least one of the parents (Photographs 24 and 25). Regenerated embryos or calli are studied with flow cytometry and isozymes in order to assess respectively their ploidy level and the origin of their nuclear genome.

Tetraploid somatic hybrids have already been selected for more than twenty interspecific and intergeneric (*Citrus* + *Fortunella* and *Citrus* + *Poncirus*) combinations. One of them (*Citrus deliciosa* + *Poncirus trifoliata*) is promising as a rootstock and it is being propagated for multilocal evaluation in the Mediterranean area. The other allotetraploid hybrids will more probably be used for triploid breeding by pollination of diploid monoembryonic mandarins.

Somatic hybridization between diploid and haploid has been done with a haploid embryogenic callus line of Clementine and nine diploid cultivars; triploid hybrids have been obtained for each combination. All these polyploid hybrids are being propagated for evaluation under Mediterranean and tropical conditions.

More attention is now given to wide intergeneric hybridization for rootstock breeding and to the development of triploid synthesis by gameto-somatic hybridization (microspore protoplast + diploid protoplasts).

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Ollitrault P., Dambier D., Cabasson C., Allent V., Engelmann F., 1995. Optimized management of citrus embryogenic calli for breeding programs. Fruits 49(5-6):394-397.

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Cabasson C., Alvard D., Dambier D., Ollitrault P., Teisson C., 1997. Improvement of citrus somatic embryo development by temporary immersion. Plant Cell Tissue Organ Cult. 50:33-37.

Ollitrault P., Dambier D., Bakry F., Auran G., Aubert B., 1997. Somatic hybridization to answer the challenge of rootstock selection for the Mediterranean basin. In: Proceedings of the Vth international congress of citrus nurserymen. 1997 Mar. 5-8. Montpellier, France (in press).

Cocoa

CIRAD has been working for five years on the development of somatic embryogenesis (Photograph 7) for two main purposes:
– first, as a reliable technique for mass propagation of hybrids to replace more traditional techniques of propagation, such as seed garden, cutting, and grafting, that are not trouble-free;
– secondly, as a mean to initiate genetic manipulation, particularly to introduce insect resistance.

Somatic embryogenesis

Since the first studies on somatic embryogenesis in 1975, only floral explants, particularly staminodes, have had some success. However, these studies have not been fully satisfactory in terms of reproducibility and response of the genotypes. Therefore, it has been decided to initiate studies with leaf explants concurrently.

So far, it is possible to define the proper leaf stage and culture conditions allowing a good callogenesis whatever the genotype and to obtain and maintain in liquid media cell suspensions with embryogenic characteristics.

The current studies focus mainly on improving the process on the basis of histological observations. This will serve for confirming the embryogenic nature of calli on solid medium and of cell suspension in liquid media before working further on the regeneration phase.

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Coconut palm

The coconut palm (*Cocos nucifera* L.) is a major agricultural crop in intertropical areas. Its importance is due to its role in oil production; it also provides cash and subsistence to small holders. The coconut sector has however several problems which affect its productivity, particularly the use of unimproved planting material, the old age of existing plantations and various pests and diseases, for which no chemical treatment is currently available. Since the coconut palm is generally cross-pollinated and heterozygous, propagation by seeds gives rise to great variability in hybrid progenies. *In vitro* vegetative multiplication of high performance individuals thus remains the only short and medium term hope for the production of homogenous planting material and for a substantial improvement in the productivity of plantations. Apart from the generation of planting material with higher yields at an earlier age, cloning would also allow rapid multiplication of selected individuals that exhibit resistance or tolerance to important diseases and to adverse growing conditions such as drought. However, coconut is a highly recalcitrant species in *in vitro* culture.

Clonal propagation by somatic embryogenesis

Since 1991, studies have been carried out by CIRAD in collaboration with ORSTOM to provide a better understanding of the causes behind the stalling or deviations often seen in coconut somatic embryogenesis. Solid progress for the mastery of coconut regeneration has been made in the last 5 years by the CIRAD-ORSTOM group and an experimental protocol is now available.

The work is presently focused on embryo germination and the establishment of plantlets in the greenhouse which is still a bottleneck. Contacts have been established with others labs in coconut growing countries to assess the performance of the protocol on a larger scale and to adjust the procedure accordingly if necessary, in collaboration with the research laboratory in Montpellier.

Selected publications

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CURRENT SPECIFIC RESEARCH CONTRACTS

Coconut: development of methods for the clonal propagation of elite, disease-resistant palms by somatic embryogenesis. European Community, EC-STDIII project, 1995-1998.

Study of coconut propagation by somatic embryogenesis. Ecos project, 1997-2001.

Coffee

Somatic embryogenesis is used at CIRAD for mass propagation of heterozygous plant material (i.e. elite F_1 hybrids of *Coffea arabica* superior to cultivated varieties, selected material from *C. canephora*) and to produce cellular targets for genetic transformation assays. These are worldwide collaborative activities involving different partners: CATIE (Centro Agronómico Tropical de Investigación y Enseñanza, Costa Rica), Promecafé (Programa Cooperativo Regional para la Protección y Modernización de la Caficultura, central America and the Caribbean), Nestlé (France) and the government of Uganda.

Somatic embryogenesis

CIRAD started by contributing to the optimization of small scale production techniques: *in vitro* propagation of microcuttings and somatic embryogenesis (Photographs 8 and 9). Expensive and sophisticated bio-reactors of other research groups did not allow for obviating the use of solid media and thus reducing production cost. Today, research at CIRAD has led to a high performance somatic embryogenesis procedure applicable to all species and genotypes at the industrial level. This procedure is based on the high frequency generation of embryogenic calli on solid media followed by cell suspension and regeneration in liquid media. This technique is being transferred to central America and Uganda.

At present, researchers at CIRAD are able to mass propagate all the genotypes of the two cultivated species, *C. arabica* and *C. robusta.* On a routine basis, embryogenic calli are generated and long term *in vitro* proliferations are obtained in cell suspensions. Large scale somatic embryogenesis propagation is now projected, thanks to a couple of improved procedures: a new temporary immersion technique for liquid media cultures and a technique allowing for the direct transfer of somatic embryos to the nursery for plantlet growing and hardening. Thus, the whole regeneration process is maintained in liquid media, starting from the embryogenic aggregates in cell suspensions up to the hardening of the somatic embryo in the same container. A first evaluation in central America yielded that the cost of a nursery plantlet produced *in vitro* is similar to the cost of producing a seedling. CIRAD uses this new procedure in Costa Rica, central America and eastern Africa.

In Costa Rica, Promecafé has initiated two years ago a genetic improvement program of *C. arabica* in central America. The objectives are to mass produce and distribute two types of elite material:

– at the international level in central America, selected F_1 hybrids stemming from crosses between the best performing cultivated varieties with wild-type accessions of Ethiopia and the Sudan. A performance trial in multiple environments is being established. This assay implies 30000 plants, 30 regional spots and 6 countries. Up to 6000 plants have already been distributed. In order to foster the development of biotechnology in central America, a network centered at CATIE is relying on four *in vitro* culture laboratories (focusing on coffee) in Guatemala, San Salvador, Costa Rica and Honduras;

– at the national institutional level, the parents of a hybrid variety, Nemaya, which is resistant to nematodes and used as a rootstock. Up to 15 000 plants have already been distributed to establish seed gardens. The diffusion of this variety will be completed in July 1998 with a total of 30000 hardened plantlets produced. This production is now supported by the national institutes where the somatic embryogenesis technique has been transferred. In parallel, conformity assays implying several thousand plants from *C. arabica* produced by somatic embryogenesis have been established, starting in 1997. No problem linked to somaclonal variation has been noticed so far.

In central America, the first plants grafted onto the Nemaya variety will be distributed to producers in the year 2000. In 2002, the best F_1 varieties will start to be commercially produced via somatic

embryogenesis. It is projected that this will produce a 30% increase of productivity in central America. If this holds true, somatic embryogenesis will allow to reduce a 30-year breeding cycle down to a mere 10 years.

In eastern Africa, in Uganda, a fully equipped *in vitro* culture laboratory has been established with funds from the European Union. The somatic embryogenesis technique initially set at CIRAD has been successfully transferred to this laboratory. It is projected that up to 15 000 plants from *C. canephora,* produced by somatic embryogenesis, will be planted in the fields in 1999.

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Van Boxtel J., Berthouly M., 1996. High frequency somatic embryogenesis from coffee leaves: Factors influencing embryogenesis, and subsequent proliferation in liquid medium. Plant Cell Tissue Organ Cult. 44:7-17.

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Berthouly M., Etienne H., 1998. Somatic embryogenesis in coffee. In: Jain S.M., Gupta P.K., Newton R.J., editors. Somatic embryogenesis in woody plants. Dordrecht, Kluwer Academic Publishers (in press).

Specific research contracts

Regional project of coffee genetic improvement in central America. Promecafé, CATIE, French cooperation (CIRAD, ORSTOM, French Ministry of Foreign Affairs), 1992-2000.

Genetic transformation

A cooperation between CIRAD and Nestlé research center in Tours, France, was initiated in 1994 on genetic transformation of coffee for resistance to coffee leaf miner *Perileucoptera coffeella.* Genetic transformation of coffee was first achieved by Nestlé (1992) with the introduction of the *gus* gene via *Agrobacterium rhizogenes,* whereas CIRAD proved that the *cryIA(c) Bacillus thuringiensis (Bt)* gene produced efficient endotoxin against the leaf miner. A collaborative project was started in order to build an efficient methodology for transformation of both species (*C. canephora* and *C. arabica*) via *Agrobacterium tumefaciens.* The contribution of CIRAD rests on a wide experience on coffee germplasm, quantitative genetics and mastering of somatic embryogenesis for both species.

So far, plasmids for genetic transformation via *Agrobacterium* have been constructed by CIRAD with a *Bt* gene, the *gus* gene and a gene for resistance to a herbicide (chlorsulfuron). These plasmids contain native or synthetic *cryIA(c)* genes.

More than 120 independent transformation events have been obtained for one genotype of *C. canephora* with the synthetic *Bt* gene. All these events allowed the regeneration of transgenic plantlets. Most of them are in greenhouses and molecular analyses are in progress before field trials.

Only few primary transformants have been obtained on two genotypes of *C. arabica.* Experiments are going on for obtaining at least 100 primary transformants for each genotype.

All the transgenic plantlets are studied by PCR and southern and western blotting. They will be analyzed for the integration of genes of interest into the genome and the production of endotoxin in the leaves for the resistance to the leaf miner. Bioassays with insects will be carried out at CIRAD in Montpellier for the evaluation of the level of resistance.

Transformed plants will then be cultivated in French Guyana, where leaf miner is present. In this first trial, the agronomical value of transformed coffees and their level of resistance to coffee leaf miner in natural conditions will be evaluated.

Genetic transformation of coffee via *Agrobacterium tumefaciens* can now be considered as efficient and introduction of other genes of interest is possible. For example, if genes against berry borer are identified, transformation for resistance to this pest will be of great interest for all the producing countries.

Selected publications

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Current specific research contract

Genetic transformation of coffee via *Agrobacterium* in order to introduce *Bt* genes against coffee leaf miner. Contract CIRAD-Nestlé.

Cotton

Cotton (*Gossypium hirsutum* L.) has attracted much interest in the field of gene transfer with the aim of introducing agronomically interesting new traits. Various areas are receiving attention. These include fiber quality, stress tolerance, fungal resistance. Cottonseed protein and oil quality is also a potential field of interest. But the areas that have progressed the most are those of herbicide and insect resistance.

Insect resistance is one of the most important goals for cotton improvement. The level of production losses due to insect damage is such that cotton protection represents about 24% of the world insecticide market. CIRAD has undertaken, within the framework of a collaboration with INRA, a research program on the genetic engineering of cotton in view of creating insect-resistant varieties.

Gene transfer

An *Agrobacterium tumefaciens*-mediated transformation procedure has been developed using a cultivar which offers the best regeneration potential. The approach adopted is to transfer the gene of interest into Coker varieties, the subsequent introduction into agronomically interesting cultivars being done through backcrosses.

The regeneration of cotton is obtained through somatic embryogenesis. The process developed by CIRAD-INRA is based on the use of hypocotyl fragments obtained from aseptically grown seedlings. Up to 80% of the hypocotyl fragments can give rise to embryogenic tissues which develop from a parenchymatous callus.

These embryogenic calli give rise to developing somatic embryos while continuing to proliferate on a hormone-free medium.

The inoculation of cotton cells by a strain of *A. tumefaciens* carrying the genes of interest is done on hypocotyl explants. Several *A. tumefaciens* strains have been tested, and the best results, in term of explants developing calli which grow on the selective medium, reach more than 60% after 40 days of culture. The timing for inoculation and coculture was determined, as well as the conditions for the selection of transformed cells, on a kanamycin-containing medium (Photograph 10). Small calli originating from vascular bundle cells appear after about one month. After several subcultures, 10 to 20% of these calli give rise to embryogenic tissues after at least 5 months of culture (Photographs 11 to 13). Studies have been undertaken on the induction of somatic embryogenesis and several modifications of the medium lead to an increase in the percentage of calli giving rise to somatic embryos, and a reduction in the delay necessary to obtain these future plantlets. At the PGEU (Plant Genetic Engineering Unit) of Kasetsart University in Thailand, with whom CIRAD collaborates, the regeneration and transformation of a local variety has been achieved (Photographs 14 to 16).

Studies aimed at optimizing the process for the regeneration of transformed plants are on going, and it is envisaged to develop alternative gene transfer methods, by transforming meristems or embryogenic tissues. In addition, the culture of cotton protoplasts has been undertaken with the aim, on the one hand, to optimize culture media and, on the other hand, to attempt direct gene transfer.

The molecular analysis of the transformed plants has shown that, with the process used, plantlets regenerated from one embryogenic line were clonal as far as gene integration is concerned. This fact is quite important for the management of transgenic material for subsequent breeding.

Transfer of genes coding for entomopathogenic proteins

The first strategy was to transfer genes from *Bacillus thuringiensis (Bt),* which produces entomopathogenic molecules and is used as a biopesticide. The integration of genes encoding entomopathogenic proteins from *Bt* has made it possible to obtain cotton lines resistant to several carpophageous insects. The ultimate goal is to obtain a durable protection, which requires in particular the stability of gene expression during the course of selfing or backcrosses, and also requires reducing the probability of the appearance of resistant insects. These concerns have led the work on the genetic engineering of cotton for insect resistance.

Bt genes exhibit a low level of expression which is due to their bacterial origin. Thus, constructs carrying synthetic genes have been developed. Modifications of the coding sequence are accomplished by exchanging codons according to the plant codon usage, as well as removing all DNA sequences potentially causing translation termination and RNA instability. A synthetic *cryIC* gene has been constructed and transferred into tobacco and cotton. The transfer into tobacco is mainly used to analyze the level of expression of the synthetic gene and to test its efficiency. The CryIC toxin is active against *Spodoptera* spp., which cause important production losses especially in several countries in South America. In collaboration with PGEU in Thailand, the synthetic *cryIA(b)* and *cryIA(c)* genes are used to create varieties resistant to *Heliothis armigera,* which is the key pest in Asia and Africa.

In addition to the problems of *Bt* gene expression, the second main concern is the appearance of resistant insects. In order to reduce this risk, several strategies have been proposed. One way is to associate, in the same plant, two genes encoding entomopathogenic proteins with different modes of action. In this respect, we have initiated studies on the use of protease inhibitor (PI) genes. Two types of PIs were studied, the soybean serine protease inhibitor CII, and the rice cysteine protease inhibitor OC-I. Bioassays were conducted on *Anthonomus grandis* (boll weevil), a Coleoptera which causes very severe damages in cotton crops in North and South America, and on *H. armigera.* The first results have shown that the incorporation of OC-I (produced in *E. coli*) in diets based on cotton tissues (leaves or bolls) induces a reduction in *A. grandis* larval growth. The Bowman Birk Inhibitor (BBI), which is similar to CII, has deleterious effect on both *A. grandis* and *H. armigera.* Transgenic cotton plants carrying either an OC-I or a CII gene have been produced. Studies aimed at evaluating the interest of using PI genes, in particular to control *A. grandis* against which no active *Bt* toxin is known, are on going.

In order to limit the selection pressure, it is envisaged to reduce the expression of *Bt* genes to certain organs, and in particular to the bolls. The search for such tissue-specific promoters has been undertaken using the technique of promoter tagging and the *Arabidopsis thaliana* T-DNA insertion mutant collection established at INRA.

Through various collaborations, the field management of transgenic insect-resistant varieties will be studied in a multidisciplinarity approach involving biotechnologists, entomologists and breeders.

A diversification of the cotton genetic engineering program is being undertaken. This includes the control of sucking pests, in particular *Aphis gossypii.* CIRAD is in the process of defining relevant research goals through interactions with various INRA researchers involved in this field. CIRAD is also interested in the search for novel sources of resistance. Finally, the study of cotton fiber cell development is being developed. This research is undertaken together with research teams who study cell elongation in the model plant *A. thaliana,* and is part of a genome mapping effort recently undertaken at CIRAD.

Selected publications

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Oil palm

The origins and causes of somaclonal variation have been intensively studied at the molecular level and the use of molecular markers for the identification of somaclonal variants and the evaluation of *in vitro* protocols is now widespread. Somaclonal variation may also be studied at the gene expression level by the differential analysis of mRNA populations.

For oil palm (*Elaeis guineensis* Jacq.), the ORSTOM-CIRAD group has developed a micro-propagation process up to the pilot scale in 4 production units spread over 3 producing countries. *In vitro* vegetative propagation has led to the production of more than 1 million clonal plantlets to date. The technique has been proven by field performance on more than 2500 ha of clonal plantations.

A coherent network has been established, linking the ORSTOM-CIRAD group to several partners in basic research (universities and research institutes) on one hand, and key players in the oil palm sector in producing countries (private companies, development agencies and national agricultural research institutes) on the other hand.

Molecular search for mRNA and genomic markers of somaclonal variation

In order to improve the reliability of the clonal process developed for oil palm, we are attempting to identify mRNA and genomic markers for screening of the "mantled" flowering abnormality found in typically 5-10% of regenerated palms. The abnormality, which is unstable over a period of years, is characterized by an altered floral organ identity in whorls 2 and 3.

Using flow cytometry, it was demonstrated that embryogenic calli (including those producing abnormal palms), somatic embryos and regenerated normal and variant palms showed the same ploidy level.

RAPD analysis was found to be efficient for distinguishing oil palm clonal lines of different genotypic origin, but failed to reveal any polymorphism associated with either mantled somaclonal variants or with the overall tissue culture process. Even with the use of more than 380 10-mer primers, the portion of the genome analyzed (approximately 0.04%) using the RAPD technique is clearly insufficient for detecting discrete genetic events that could be linked to somaclonal variation in oil palm. An AFLP-based approach is now being developed in order to assess genomic changes that could be related to somaclonal variation.

Global genomic levels of DNA methylation—(5mdC) / (5mdC+dC)—have been investigated in regenerated oil palms, with the aim of comparing mother palm/regenerants and normal/variant regenerants of the same clonal line. Global levels of genomic DNA methylation in oil palm reached 25%, in agreement with levels already observed in other plants. The measured levels of DNA methylation did not discriminate the mantled variants at the adult stage. Nevertheless, this approach will be useful for the monitoring of genetic conformity throughout the *in vitro* culture process. In order to investigate patterns of DNA methylation, we are using RFLP in conjunction with oil palm cDNA probes and isoschizomeric restriction enzyme pairs, showing differential sensitivity to the methylation of dC residues (e.g. *Mspl*/*Hpa*II).

We are now developing a novel approach based on the analysis of differential genome expression in normal/variant plant material. This approach is centered on techniques available to study differences in the abundance of specific mRNA species between populations. We are using the PCR-based differential display method in order to characterize gene expression in calli and embryoids producing normal and abnormal plants, so that to identify an early marker of the mantled phenotype.

The ORSTOM-CIRAD group is studying the expression patterns of "candidate" genes likely to be affected by the somatic embryogenesis-based process under study. In particular, homeotic genes of the MADS box family are under investigation, since in several higher plant species, genes of this type have been shown to display mutant phenotypes comparable to those seen in oil palm. Studies are simultaneously being carried out first on the activity of these genes in response to tissue culture, and second on their methylation state, which may influence gene expression and thereby the phenotype of regenerant plants.

Selected publications

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Rival A., Tregear J., Verdeil J.L., Beulé T., Richaud F., Duval Y., 1997. Molecular and cytological analysis of the "mantled" somaclonal variation in oil palm. In: Vth international congress of plant molecular biology. 1997 Sep. 21-27. Singapore. Poster n° 1378.

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Rival A., Tregear J., Hartman C., Verdeil J.L., Beulé T., Richaud F., Rode A., Duval Y., 1998. Molecular search for mRNA and genomic markers of the oil palm "mantled" somaclonal variation. Acta Hort. (in press).

Research contract

Somaclonal variations in oil palm: molecular analysis of *in vitro* cultures producing normal and abnormal plants. ORSTOM-CIRAD, IBP-CNRS-University of Paris XI, PORIM. Sponsor, PORIM (Palm Oil Research Institute of Malaysia), Kuala Lumpur, Malaysia, 1997-2000.

Rice

In rice, zygotic embryos and microspores are the most widely used tissues for inducing embryogenic calluses which are materials for establishing embryogenic cell suspensions which in turn are used for isolating protoplasts able to regenerate plants (Photograph 23). Epithelial and subepithelial scutellum cells of the embryo, homogeneous cell clumps grown in suspension and preparations of isolated protoplasts are suitable targets for transformation. Rice is the only cereal where transgenic plants have been repeatedly and efficiently obtained using three transformation techniques: direct gene transfer through physical (electroporation) or chemical (polyethylene glycol, PEG) treatment of protoplasts; microprojectile bombardment to cell suspensions, embryo scutellum and scutellar callus; and transfection of immature embryos and young scutellar callus by *Agrobacterium tumefaciens*. These transformation systems have been used for generating transgenic plants resistant to various biotic and abiotic stresses and as model tools for studying promoter functions and deregulation of genes as well as fastening positional cloning.

Regeneration and assessment of somaclonal variation

Efficient plant regeneration has been achieved from embryo- and microspore-derived callus in a range of both temperate and tropical japonica cultivars of *Oryza sativa* and its African perennial wild relative *Oryza longistaminata*. Emphasis was placed on understanding the regeneration pathways and differences of regenerability among genotypes using histological analyses. Both somatic and germinal tissue-derived calluses have been used to establish embryogenic cell suspensions from which regenerable protoplasts have been prepared. The various varieties tested have exhibited a range of response to the protoplast-to-plant technique namely in frequency of plant regeneration and of formation of albino plants. Frequency of albino plants was generally higher when microspore calluses were used for initiating the cell suspension.

Microspore callus-derived protoplasts generated plants exhibiting ploidy levels ranging from n to 6n including 50 to 60% diploid plants whereas 75% of plants regenerated from embryo callus-derived protoplasts kept their original ploidy level. Changes of ploidy level are likely due to spontaneous polyploidization of the protoplast genome during the early phases of culture. Field evaluation of progenies of 110 and 75 diploid plants derived from protoplasts of variety Miara and Ariete respectively have demonstrated occurrence of significant changes for agronomical characters. The frequency, direction and range of variation appeared genotype-dependent. These variations, apparently fixed, may have in some cases an agronomical interest. Contrastingly, RAPD markers did not detect any change generated by the tissue culture procedure in the same materials.

Selected publications

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Guiderdoni E., Chaïr H., 1992. Plant regeneration from haploid cell suspension-derived protoplasts of Mediterranean rice (*Oryza sativa* cv. Miara). Plant Cell Rep. 11:618-22.

Alemanno L., Guiderdoni E., 1994. Increased doubled haploid plant regeneration from rice (*Oryza sativa* L.) anthers cultured on colchicine supplemented media. Plant Cell Rep. 13:432-436.

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Mezencev N., Ghesquière A., Marmey P., Combes M.C., Guiderdoni E., 1997. Assessment of RAPD markers to detect genetic change in protoplast-derived rice plants. J. Genet. Breed. 51:97-102.

Gene transfer

Transgenic rice plants have been efficiently generated through PEG-mediated direct gene transfer to protoplasts of several cultivars. However, most of the plants exhibited sterility, as reported by other research groups. Abnormal ploidy was found to be the main source of sterility. From 1995, a method based on microprojectile-mediated gene transfer to seed embryo-derived embryogenic nodular units has been set up and used to routinely generate transgenic plants in several japonica cultivars with efficiencies ranging from 5 to 25% in term of independent transformation events produced per bombarded embryogenic nodule. More than 2000 transgenic plants have been produced through this procedure by 1997. *Agrobacterium*-mediated transformation has also recently been used to generate transgenic plants and the procedure is being optimized on elite cultivars to serve as the preferred routine transformation technique in two projects funded by the European Commission.

Both protoplast and microprojectile-based gene transfer techniques have been used to transfer genes of interest (*cryIA(c)* and *cryIB Bacillus thuringiensis* endotoxin genes, *pat* gene). Striped stemborer (*Chilo suppressalis* L.) and ammonium glufosinate resistant lines of elite rice cultivars have been successfully generated. Field trials of selected materials are scheduled by 1999. Gene transfer to model rice genome is also used to carry on functional analyses of tissue-specific and/or wound-inducible promoters of rice, wheat, maize and viral origins. Large scale production of transgenics will also be achieved in the framework of an European Community funded project to participate in the generation of a core population of integration events of various constructs containing non autonomous maize transposable elements and gene-promoter traps, spanning the whole rice genome, which will serve as transposon launching pads for directed mutagenesis and detection of genes located in their vicinity.

Selected publications

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Current research contracts

Engineering rice for resistance to insects. European Commission Fair, n° 97 3761.

Rice transposon mutagenesis for the identification of agronomically important genes in cereals. European Commission, Biotech, n° 96 2132.

Genetic engineering of salt and drought tolerance in rice cultivars. European Commission, Inco 97-3147.

Rubber tree

The future of natural rubber production is tied to a strong increase in plantation productivity. Biotechnologies offer the fastest way of improving planting material quality through cloning by somatic embryogenesis, the breeding of genotypes on their own roots and the creation of genetically transformed genotypes.

Somatic embryogenesis and genetic transformation

For the first time in *Hevea,* several embryogenic callus lines have been obtained for commercially worthwhile genotypes. They have been maintained in their proliferation phase for several years (Photographs 4 and 5). An effective regeneration procedure was used to produce more than 6000 *in vitro* plantlets, for clone PB260, in 1997. The plantlets were sent to different overseas partners for planting in trials—IDEFOR-DPL (Institut des forêts, Côte d'Ivoire), Michelin, RRIT (Rubber Research Institute of Thailand). The first trials, planted in 1992, have given growth results that are markedly in favor of *in vitro* plantlets (Photograph 6). Research to develop a technique for genetic transformation by *Agrobacterium* was launched in 1996 with the view of understanding the mechanisms of latex production. The study will lead on to the selection of candidate genes for the creation of transgenic *Hevea* trees with higher rubber yields. The first encouraging results have been obtained by integrating T-DNA and obtaining the expression of *gus* activity more than two months later in groups of proliferating cells.

Research is now geared towards genotype diversification and improvement of somatic embryogenesis yields with a view to mass production. The results are validated on an ongoing basis through the production of *in vitro* plantlets for planting in field trials in conjunction with partners. These results are also used for developing an effective and stable genetic transformation technique for the reproducible regeneration of transformed plants (Kasetsart University, Thailand). It will be possible to use such a technique to isolate- and test-specific promoters.

Selected publications

Carron M.P., Campagna S., Chaine C., Etienne H., Lardet L., Leconte A., 1995. Somatic embryogenesis in rubber (*Hevea brasiliensis* Müll. Arg.). In: Jain S.M., Gupta P.K., Newton R.J., editors. Somatic embryogenesis in woody plants. Dordrecht: Kluwer Academic Publishers, p. 117-136.

Montoro P., Etienne H., Carron M.P., 1995. Effect of calcium on callus friability and somatic embryogenesis in *Hevea brasiliensis* Müll. Arg.: relations with callus mineral nutrition, nitrogen metabolism and water parameters. J. Exp. Bot. 46(283):255-261.

Carron M.P., Dea B.G., Tison J., Leconte A., Keli J., 1997. Croissance en champ de clones d'*Hevea brasiliensis* produits par culture *in vitro.* Plant. Rech. Dév. 4(4):264-273.

Etienne H., Lartaud M., Michaux-Ferrière N., Carron M.P., Berthouly M., Teisson C., 1997. Improvement of somatic embryogenesis in *Hevea brasiliensis* immersion technique. In Vitro Cell Dev. Biol. Plant 33:81-87.

Perrin Y., Doumas P., Lardet L., Carron M.P., 1997. Endogenous cytokinins as a biochemical marker of rubber tree (*Hevea brasiliensis* Müll. Arg.) clone rejuvenation. Plant Cell Tissue Organ Cult. 47:239-245.

Current specific research contract

Experimental production of *in vitro* plantlets by somatic embryogenesis. Michelin, MENRT, France.

Gene expression in transgenic plants

Plant genetic transformation is a potential tool in different areas such as manipulation and understanding of biochemical processes, knowledge of genome regulation and integration of genes which cannot be manipulated by classical breeding.

Control and regulation of transgene expression are required for gene transfer in transgenic plants. This regulation can occur during different steps of gene expression but more particularly during transcription. The promoters ensure this control. Promoters with spacial and temporal expression patterns are being studied at CIRAD in order to investigate and ultimately manipulate responses to biotic and abiotic stresses. The plants used include rice and cotton, that can be considered as references for monocots and dicots, as well as rubber tree, which has particular targets for gene expression control.

For rice and cotton, the objectives are to characterize inducible and/or tissue-specific promoters in order to achieve a better control of the expression of pest resistance genes. Wound inducible and/or fungal pathogen responsive promoters were derived from rice *ltp* gene (lipo transfer protein), *hrgp* (hydroxyproline-rich glycoprotein) and *mpi* (maize protease inhibitor) genes from maize. Vascular tissue-specific promoters were isolated from MSV (maize streak virus) and *Arabidopsis thaliana* whereas fruit- and seed-specific promoters were derived from wheat and *A. thaliana.* Expression patterns of these promoters and a set of deleted versions, driving the *gus* reporter gene are being monitored in homo- and/or heterologous plants—rice, tobacco, *A. thaliana* (Photographs 26 to 28). Some of them are already used in genetic engineering assays (essentially *Bt* transcriptional fusion constructs).

For rubber tree, the aim is to make use of ethylene inducible and/or latex-specific promoters to modify some metabolic pathways of laticiferous cells. One application can be to delay latex coagulation by under-expression of hevein or its receptor; another application can be to confer a better tolerance to oxidative stresses when trees are under intense conditions of exploitation. At present, a hevein gene promoter (latex cell-specific) and glutamin synthase gene (ethylene-inducible) promoters have been partially cloned. Functionality studies of those promoters are projected.

Selected publications

Cordero M.J., San Segundo B., Delseny M., Guiderdoni E., 1997. Activity of the promoter of the wound-inducible maize protease inhibitor gene in transgenic rice. In: General meeting of the international program on rice biotechnology of the Rockefeller Foundation. 1997 Sep. 15-20. Malacca, Malaysia.

Digeon J.F., Guiderdoni E., 1997. A 388bp region of a wheat puroindoline gene promoter is sufficient to direct tissue-specific expression in transgenic rice seeds. In: Vth international congress of plant molecular biology. 1997 Sep. 21-27. Singapore.

Legavre T., Guiderdoni E., Peterschmitt M., Michaux-Ferrière N., Teisson C., 1997. Leftward transcriptional activity of the maize streak virus promoter: Vascular tissue specificity in transgenic rice. In: Vth international congress of plant molecular biology. 1997 Sep. 21-27. Singapore.

Montoro P., Pagant S., Giband M., Michaux-Ferrière N., Triaire L, Tourneur J., Mollier P., Bechtold N., Voisin R., Pannetier C., Chupeau Y., Pelletier G., 1997. Trapping and isolation of a phloem-specific promoter from *Arabidopsis thaliana.* In: Vth international congress of plant molecular biology. 1997 Sep. 21-27. Singapore.

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Pujade-Renaud V., Montoro P., Phuangkosol N., 1997. Cloning of latex-specific and/or ethylene-inducible promoters from rubber tree. In: Seminar-workshop on the biochemical and molecular tools for exploitation diagnostic and rubber tree improvement. 1997 Oct. 20-22. Bangkok, Thailand.

Pujade-Renaud V., Perrot-Rechenmann C., Prevot J.C., Lacrotte R., Guern J., 1997. Characterization of a full length cDNA clone coding for glutamine synthetase from rubber tree latex. Plant Physiol. Biochem. 35:85-93.

Bacillus thuringiensis insecticidal proteins

Bacillus thuringiensis makes up to 98% of all biopesticides and represents the quasi exclusive source of pest-resistance genes for the development of transgenic plants (insects and nematodes). The current fast development of pest-resistant transgenic crops producing *B. thuringiensis* toxins illustrates their high potential and interest in the development of environmentally-friendly means of pest control. Indeed, these proteins cumulate several advantages such as a high specificity, a short life in the environment, a high and fast toxic activity and a track record of more than 40 years of toxicological and ecotoxicological analyses which consistently demonstrated their safety to non-target organisms, and especially to mammals. It is expected that *B. thuringiensis* toxin genes will continue to be the most important source of pest-resistance genes in the transgenic plants to be developed during the next decade and that the demand for *B. thuringiensis*-related products (sprayable biopesticides and transgenic plants) will increase. There is, however, a need for new insecticidal proteins to overcome problems related to the use of these products which are the narrow host range related to the high specificity and the potential for the development of resistant populations of pests.

Researchers at CIRAD have been working on *B. thuringiensis* for the last six years. Aspects addressed within this project are:
- identification and isolation of novel genes;
- establishment of collections of native and synthetic toxin genes;
- production of a large panel of pure toxins from recombinant strains;
- analysis of the mode of action and of its impact on broad insect resistance;
- development of alternative approaches for *in vitro* assessment of toxins activity;
- analysis of toxin/receptor interactions and of their impact on specific insect resistance.

Toxins active against several tropical pests of maize, rice and coffee have been identified. Constructs optimized with respect to insect control and resistance management have been developed and insect-resistant transgenic maize, rice and coffee have been obtained. Transgenic maize resistant to African and American stemborers have been developed by CIMMYT using optimized constructs developed at CIRAD. A new PCR-based strategy allowing detection of novel genes has been developed. Novel strains as well as novel insecticidal and nematicidal toxin genes have been isolated and characterized and novel mechanisms of crystal formation have been described. Results have also been obtained on mechanisms of resistance, on mode of action of toxins and structure-function relationships. Current works are devoted to the isolation of novel genes, the understanding of the mode of action and the development of constructs optimized for the control of various coffee, sugarcane, cocoa, sorghum and banana pests (i.e. Lepidoptera, Coleoptera and nematodes).

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Itoua-Apoyolo C., Drif L., Vassal J.M., De Barjac H., Bossy J.P., Leclant F., Frutos R., 1995. Isolation of multiple subspecies of *Bacillus thuringiensis* from a population of the European sunflower moth, *Homoeosoma nebulella.* Appl. Environ. Microbiol. 61:4343-4347.

Moar W.J., Puzstai-Carey M., Van Faassen H., Bosh D., Frutos R., Rang C., Luo K., Adang M.J., 1995. Development of *Bacillus thuringiensis* CryIC resistance by *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae). Appl. Environ. Microbiol. 61:2086-2092.

Davidson E.W., Patron R.B.R., Lacey L.A., Frutos R., Vey A., Hendrix D.L., 1996. Activity of natural toxins against the silverleaf whitefly, *Bemisia argentifolii,* using a novel feeding bioassay system. Entomol. Exp. Appl. 79:25-32.

Fiuza L.M., Nielsen-LeRoux C., Gozé E., Frutos R., Charles J.F., 1996. Binding of *Bacillus thuringiensis* CryI toxins to themidgut brush border membrane vesicules of *Chilo suppressalis*

(Lepidoptera: Pyralidae): evidence of shared binding sites. Appl. Environ. Microbiol. 62:1544-1549.

Rang C., Bès M., Lullien-Pellerin V., Wu D., Federici B.A., Frutos R., 1996. Influence of the 20-kDa protein from *Bacillus thuringiensis* subsp. *israelensis* on the rate of production of truncated CrylC proteins. FEMS Microbiol. Lett. 141:261-264.

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Racapé J., Granger D., Noulin J.F., Vachon V., Rang C., Frutos R., Schwartz J.L., Laprade R., 1997. Properties of the pores formed by parental and chimeric *Bacillus thuringiensis* insecticidal toxins in planar lipid bilayer membranes. Biophys. J. 72:A82.

Rang C., Bès M., Moar W.J., Frutos R., 1997. Simultaneous production the 34-kDa and 40-kDa crystal proteins from *Bacillus thuringiensis* subsp. *thompsoni* is required for the formation of inclusion bodies. FEBS Lett. 412:587-591.

Guerreiro-Filho O., Denolf P., Peferoen M., Decazy B., Eskes A.B., Frutos R., 1998. Susceptibility of the coffee leaf miner (*Perileucoptera* spp.) to *Bacillus thuringiensis* delta-endotoxins: a model for transgenic perennial crops resistant to endocarpic insects. Curr. Microbiol. (in press).

Yuan Z., Frutos R., Pasteur N., Nielsen-LeRoux C., Charles J.F., 1998. Detection of the binary toxin genes of several *Bacillus sphaericus* strains and their toxicity against sensible and resistant *Culex pipiens*. Acta Entomol. Sinica (in press).

Current specific research contracts

CIRAD has been contracted by CIMMYT and UNDP for the last five years to identify active toxins and develop synthetic genes and optimized constructs for the creation of borer-resistant tropical maize.

A four-year European Commission project (Fair project) is now beginning for developing borer-resistant Mediterranean rice.

Consulting contracts have been signed with the private sector.

Optimized vectors for plant transformation

The recent development of transgenic plants to a commercial level as well as the increase in demand for this technology from both developing and industrial countries indicate clearly the current trends and future needs in agriculture. In tropical agronomy, and therefore in most of the developing countries, an important diversity of needs and constraints exists with respect to the number of targeted crops and varieties, geographic distribution or type of required trait genes. Addressing the issue of developing transgenic crops for tropical agronomy in an efficient and cost effective way requires the development of molecular tools and strategies allowing a fast and easy adaptation to various crops, constraints and agrosystems. These tools and strategies are needed to easily adapt any existing constructs.

CIRAD has been working on the development of flexible and optimized systems for the last four years and has created several series of plant expression vectors adapted to the transformation of either monocotyledonous or dicotyledonous plants through biolistics or *Agrobacterium.* These expression systems, or optimized vectors, are based on a cassette system allowing a quick and easy replacement of any key element such as promoter, terminator, trait gene, marker gene or resistance gene. These tools make possible the adaptation to requests from various partners and an easy evolution of a given product. These systems have already been successfully used to create transgenic rice, maize, sorghum, tobacco and coffee.

These systems have been used in conjunction with the *B. thuringiensis* project to create insect-resistant transgenic maize, rice and coffee plants. Transgenic maize resistant to African and American stemborers have been developed by CIMMYT using optimized constructs developed at CIRAD. Similarly insect-resistant coffee and sorghum have been obtained in collaboration respectively with Nestlé (France) and University of Queensland (Australia). Transgenic rice resistant to the striped stemborer have been created as an internal CIRAD project.

The development of multivalent, adaptable and flexible molecular tools for transfer and expression of foreign genes in plants is located downstream from other projects aiming at isolating novel trait genes such as the *Bacillus thuringiensis* project presented elsewhere. The objective is now to develop constructions adapted to the control of important pests on several tropical crops which will both carry combinations of *B. thuringiensis* toxin as well as potential for product evolution and for resistance management. This strategy is now also extended to the control of tropical diseases through the transgenic approach.

Selected publications

Breitler J.C., Marfa V., Royer M., Vercambre B., Legravre T., Frutos R., Altosaar I., Guiderdoni E., 1997. Striped stemborer-resistant Mediterranean rices through transfer of *Bacillus thuringiensis cryIA(c)* and *cryIB* synthetic genes. In:Vth international congress of plant molecular biology. 1997 Sep. 21-27. Singapore.

Current specific research contracts

In addition to *B. thuringiensis*-related contracts, CIRAD has been contracted by CIMMYT and UNDP for the last three years to develop optimized constructs for the creation of borer-resistant tropical maize.

A four-year European Commission project (Fair project) is beginning for developing borer-resistant Mediterranean rice.

Supporting research

Population structure of tropical phytopathogenic fungi, bacteria and nematodes

Studying population structure of pathogens has always been a major topic in plant pathology at CIRAD in connection with resistance breeding programs. Knowledge of pathogen population structures is essential both to resistance breeding efforts and to development of strategies for the deployment of resistance. Thus, the main objectives of these studies are i) to evaluate the amount and the distribution of pathogenic and genetic variation, ii) to understand the evolutionary mechanisms by which pathogen populations change. Pathogen population studies are conducted using molecular markers and inoculations of plants under controlled conditions (pathogenicity tests). The major studied pathogens and results obtained at CIRAD are listed in the table I. Until now, population structures were mainly analyzed in space with samples from different geographical origins. In order to predict efficiency and durability of resistance deployment strategies, further studies of pathogen population structure are planned concurrently with epidemiological studies in relation with host resistance.

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Carlier J., Mourichon X., Gonzáles de Léon D., Zapater M.F., Lebrun M.H., 1994. DNA restriction fragment length polymorphisms in *Mycosphaerella* species that cause banana leaf spot disease. Phytopathology 84:751-756.

Rott P., Davis M.J., Baudin P., 1994. Serological variability in *Xanthomonas albilineans,* causal agent of leaf scald disease of sugarcane. Plant Pathol. 43:344-349.

Fallas G., Sarah J.L., 1995. Effect of temperature on the *in vitro* multiplication of seven *Radopholus similis* isolates from different banana producing zones of the world. Fund. Appl. Nematol. 18:445-451.

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Jaunet T., Laguerre G., Lemanceau P., Frutos R., Notteghem J.L., 1995. Diversity of *Pseudomonas fuscovaginae* and other fluorescent Pseudomonads isolated from diseased rice. Phytopathology 85:1534-1541.

Carlier J., Lebrun M.H., Zapater M.F., Dubois C., Mourichon X., 1996. Genetic structure of the global population of banana black leaf streak fungus, *Mycosphaerella fijiensis.* Mol. Ecol. 5:499-510.

Fallas G., Hahn M., Fargette M., Burrows P., Sarah J.L., 1996. Molecular and biochemical diversity among isolates of *Radopholus similis* from different areas of the world. J. Nematol. 28:422-430.

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Roumen E., Levy M., Notteghem J.L., 1997. Characterization of the European pathogen population of *Magnaporthe grisea* by DNA fingerprinting and pathotype analysis. Eur. J. Plant Pathol. 103: 363-371.

Table I. Results obtained at CIRAD on major pathogens.

	Methods	Main results	Studies in progress
Fungi			
• *Mycosphaerella fijiensis* (black leaf streak disease of bananas)	RFLP, PCR-RFLP Inoculation	Global scale: center of diversity in Asia, differentiation between continents, high level of diversity and random mating	Continental scale structure in Africa and Latin America, pathogenic variability
• *Magnaporthe grisea* (rice blast disease)	RFLP, RAPD, SCAR Inoculation (virulence)	European scale: clonal lineages related to pathotypes, differentiation between countries Global scale: center of diversity in Asia, migrations	Global and country scale structures Existence of recombination
• *Phytophthora palmivora* (black pod of cocoa and coconut)	Isozyme Inoculation	Global scale: center of diversity in Asia, differentiation between populations from cocoa and coconut	Pathogenic variability
• *Phytophthora megakarya* (black pod of cocoa)	Isozyme, RAPD Inoculation	African scale: differentiation between West and central Africa Clonal reproduction, existence of recombination	Pathogenic variability
• *Colletotrichum gloeosporioides* (hevea anthracnose)	RAPD, PCR-RFLP Sequencing	Global scale: differentiation between populations, highest level of diversity in Asia	Taxonomy and phylogeny
• *Colletotrichum kahawae* (coffee berry disease)	Isozyme, RAPD, PCR-RFLP Sequencing, inoculation	African scale: low level of diversity, differentiation between West and East Africa	Taxonomy and phylogeny, West Africa structure, pathogenic variability
Bacteria			
• *Pseudomonas* spp. (sheath brown rot of rice)	Serology, PCR-RFLP Inoculation	Differentiation between *P. fuscovaginae* and other *Pseudomonas* species Identification of pathogenic and opportunistic groups	
• *Xanthomonas albilineans* (leaf scald of sugarcane)	Serology, PAGE, AFLP Inoculation	Global scale: identification of 3 serovars, 10 genomic groups and 3 pathogenic groups	Global scale structure Pathogenic variability
Nematodes			
• *Radopholus similis* (bananas)	Isozyme, RAPD, AFLP Inoculation	Global scale: 2 genomic groups, high diversity of pathogenicity	Global, continental and country scale structures
• *Pratylenchus coffeae* (bananas and coffee)	Isozyme, AFLP Morphology, inoculation	Populations specialized on bananas or on coffee Differentiation between populations	Taxonomy Global scale structure
• *Meloidogyne* spp. (coffee)	Isozyme, AFLP Morphology, inoculation	Latin America scale: high level of diversity and differentiation between populations	Taxonomy and phylogeny

Characterization, taxonomy and variability of plant trypanosomatids

Trypanosomatids (*Phytomonas* spp.) are specifically associated with wilts of cultivated crops in Latin America and in the Caribbean (coconut, oil palm, coffee, *Alpinia purpurata*). Morphologically identical microorganisms appear to live as symbiots in some latex bearing plants and others induce some damages to fruits and seeds (tomato, maize). CIRAD has been working on this matter since 1984. The research program has been funded by three consecutive European grants.

CIRAD was the first to isolate and *in vitro* culture, axenically, the trypanosomatids from laticifers tubes, and up to now, is the only one to grow phloem-restricted trypanosomatids associated with wilts in Latin America. A collection of about 60 isolates (53 of which are phloem-restricted), are maintained in liquid nitrogen. CIRAD in collaboration with various other research institutes —University of Bordeaux II, ORSTOM Montpellier, Institut Gustave Roussy (France), Universities of Sao Paulo, Brasilia and Rio de Janeiro (Brazil), University of Granada (Spain), University of Louvain (Belgium), Berhnard Nocht Tropical Institut (Germany)—has developed different serological and molecular tools in order to characterize these organisms. All the techniques and tools used (monoclonal antibodies and immunofluorescence, multi-locus enzyme electrophoresis, random-primer DNA typing, RAPD, and RFLP of KDNA minicircles), confirmed the heterogeneity of plant trypanosomes. They can be separated into, at least, three categories: phloem restricted, latex origin and fruit isolates. Variability within the last two categories is great. Two groups of intraphloemic isolates were found. Among plant trypanosomes, those associated with wilts in Latin America seem to be the easiest to characterize, the more so since they appeared as members of a monophyletic clade defined by a synapomorphic pattern generated by one band with one primer.

The possibility to fingerprint plant trypanosomatids will encourage studies in molecular epidemiology. These tools and techniques will be used very soon for a better understanding of trypanosomes-Pentatomide vector relationships. The long term application of these studies is an integrated control of these wilts.

Selected publications

Marche S., Roth C., Manohar S.K., Dollet M., Baltz T., 1993. RNA virus-like particles in pathogenic plant trypanosomatids. Mol. Biochem. Parasitol. 57(2): 261-268.

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Dollet M., Gargani D., Muller E., Vezian K., 1997. Mise au point d'une méthode d'acquisition de trypanosomes (*Phytomonas* spp.) cultivés *in vitro* par *Lincus croupius (Pentatomidae).* In: ANPP. IV^{e} conférence internationale sur les ravageurs en agriculture: tome III. 1997 Jan. 6-8. Montpellier, France, p. 887-893.

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Characterization and variability of plant viruses

Before the 1960s no much consideration was brought to the wide variability that could be found for a given virus (symptomatology, pathogenicity, seed transmission ratio, biological and serological properties, virus-vector-plant relationship). CIRAD and ORSTOM started studying variability of plant viruses at the end of the 1980s because it is a prerequisite to any control of virus diseases and particularly using the genetic methods. Pathogen variability is also a major concern in the quarantine activities for sugarcane and banana, in order to be able to detect all the strains of a given virus. Due to the applied mandate of CIRAD all the viruses which are studied are economically important ones due to the yield loss that they are causing or at least to their potential threat on cultivated crops. Peanut clump virus (PCV, pecluvirus) is causing a disease on groundnut which is difficult to control because it is seed transmitted; maize streak virus (MSV, geminivirus) is the most damaging virus disease on maize in Africa; rice yellow mottle virus (RYMV, sobemovirus) became during the last 20 years a problem on rice in Africa probably due to the intensification of this crop; three potyviruses of vegatitively multiplied crops are also studied, the sugarcane mosaic virus (SCMV) and the banana bract mosaic virus (BBMV) which both are quarantine pathogens, and the yam mosaic virus (YMV) which is currently characterized for its variability and its damages on yam production. Other studies are also performed on virus transmission through insects and on resistance mechanisms in plants to virus infection.

A great variability was found—in symptomatology, serology, RNA2 sequence—of the PCV, associated with important differences in the pathogenicity and seed transmission rate. Five distinctive serogroups were identified using a set of eight monoclonal antibodies.

Based on molecular studies it was shown that all the isolates which were serologically related to the YMV are belonging to the same potyvirus species despite their high variability. This variability could be related to the geographic origin of the isolates.

The investigation of potyvirus infections on banana showed that most of them are related to BBMV. A variability study is currently carried out to develop a quarantine test which will be able to detect all the potyvirus infections on banana.

The investigation of badnavirus infections on banana showed that they are related to BSV (banana streak virus). The possible introgression of the virus in the plant genome is being studied in relation with symptoms expression. This work is conducted with the University of Minnesota (USA) within the INIBAP framework.

Concerning SCMV, the assessment of sugarcane cultivar response to infection showed that symptom severity differ according to the isolate used as inoculum. These differences are under investigation using a PCR-RFLP technique.

The studies carried out with MSV are field-oriented due to the breeding program of CIRAD in which resistant maize genotypes are produced under artificial inoculation. Although MSV is considered to be a very stable DNA virus according to symptoms observed on infected plants and serological characterizations, it was found that island isolates of MSV are genetically distinct from continental African isolates. The severity of the isolate used to assess maize cultivar response to infection was increased by serial passages on partially resistant maize, and resulted in an isolate which was shown to be composed of several subpopulations differing by a few nucleotides. This experiment gave information on how to maintain a screening isolate and on the risk of resistance breakdown. A molecular and biological change was detected on a virus population after a host change (maize to *Coix lacryma-jobi*) showing the impact of wild hosts on viral epidemics.

Like for MSV the RYMV studies are connected with a breeding program for resistance. Based on serological and molecular studies focused on the capsid protein, it was shown that the variability of this virus has to be taken into account for the breeding work.

These works led to collaborations with several laboratories: IBMP-CNRS (France), John Innes Centre (UK), ILTAB (International Laboratory for Tropical Agriculture Biotechnology, USA).

All the studies described above are aiming to control viral diseases either by a better understanding of viral epidemics, or by contributing to the production of resistant plants obtained by breeding, by engineering or by limiting the spread of viruses through quarantine work. The biological variations associated to the molecular variability will be studied. Knowing that variability is needed for viruses to adapt to new environmental conditions, the ability of the viruses to adapt to partially resistant cultivars and the influence of the wild hosts will be monitored in controlled conditions. Characterization of undescribed viruses is still needed in tropical countries. The improvement of the diagnosis tests either for detecting new pathogens or for improving the specificity and sensitivity of existing tests is a constant concern of the quarantine work.

Selected publications

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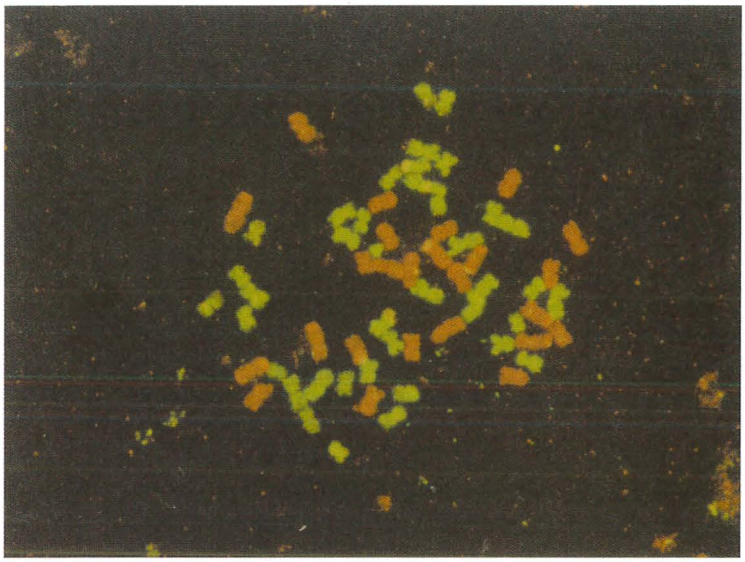
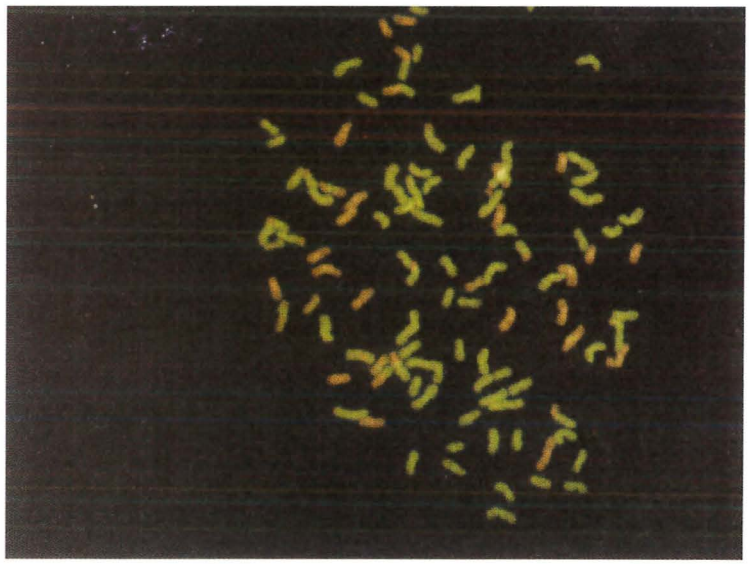
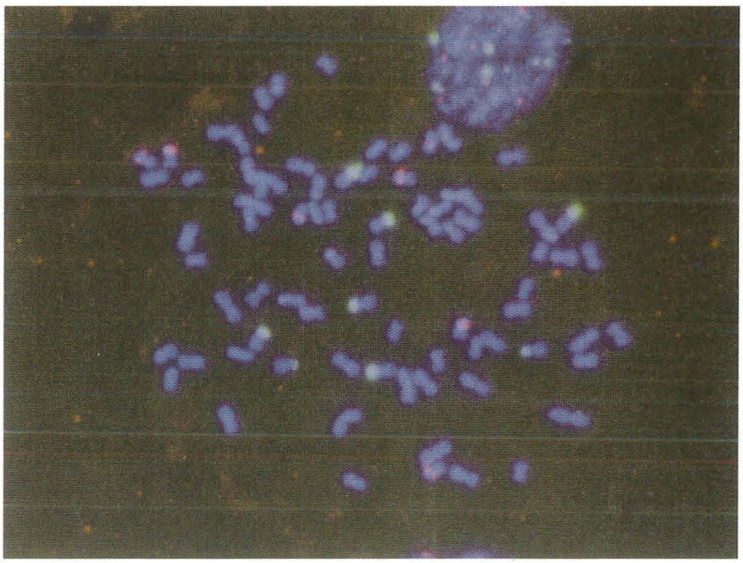


1. Chromosomes of an intergeneric hybrid between S. officinarum *and* Erianthus arundinaceus *after genomic* in situ *hybridization using* S. officinarum *total DNA detected in green and* E. arundinaceus *total DNA detected in red.*

2. Chromosomes of the sugarcane cultivar Nco 376 after genomic in situ *hybridization using* S. officinarum *total DNA detected in green and* S. spontaneum *total DNA detected in red.*

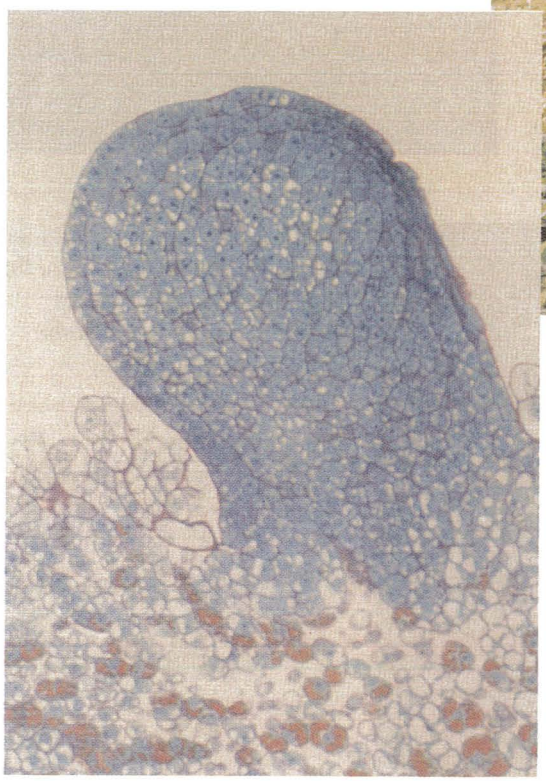




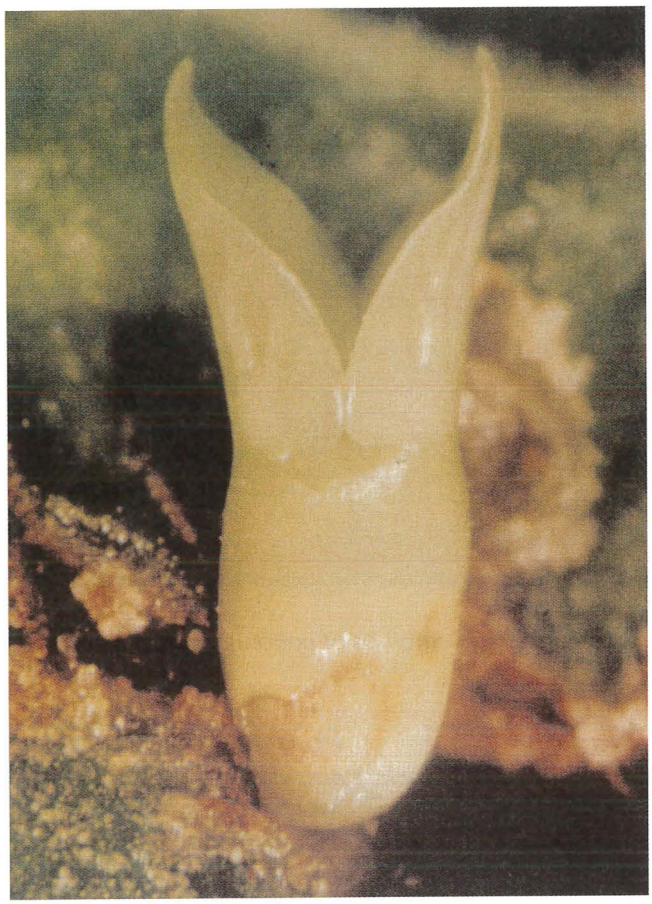
3. Chromosomes of S. officinarum *after* in situ *hybridization of the 18S-5.6S-26S rDNA genes detected in green and of the 5S rDNA genes in red.*

4. Rubber tree somatic embryo.

6. Rubber trees from somatic embryos in field trials in Côte d'Ivoire.

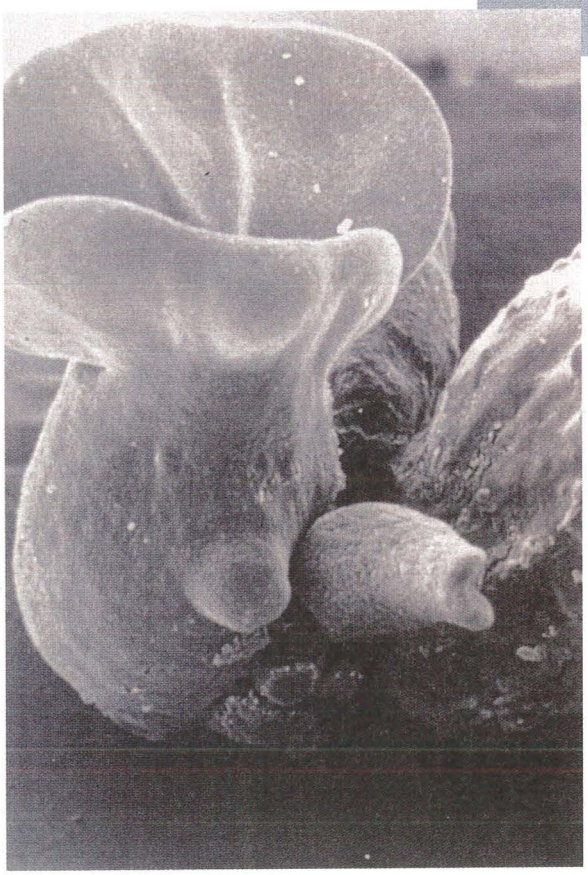


5. Rubber tree somatic embryo.

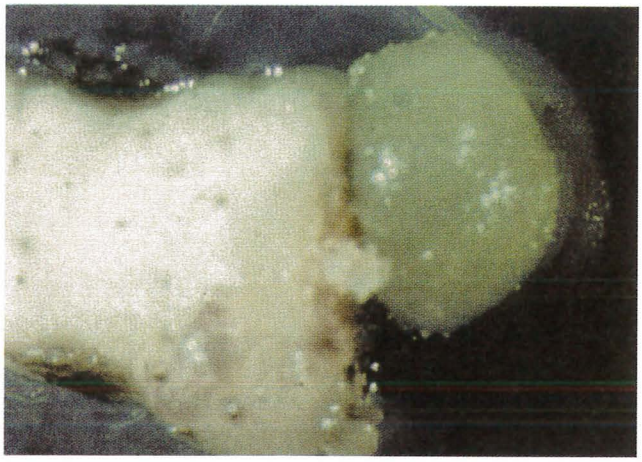


7. Cocoa somatic embryo.

8. Coffee microcuttings.

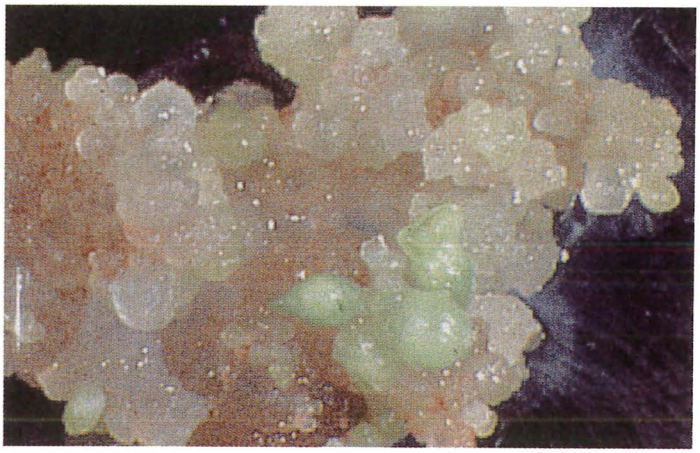


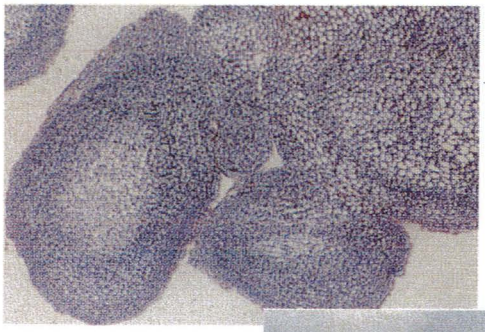
9. Coffee somatic embryo.



10. Transformed callus of cotton on a selective culture medium.

11. Embryogenic callus of cotton.

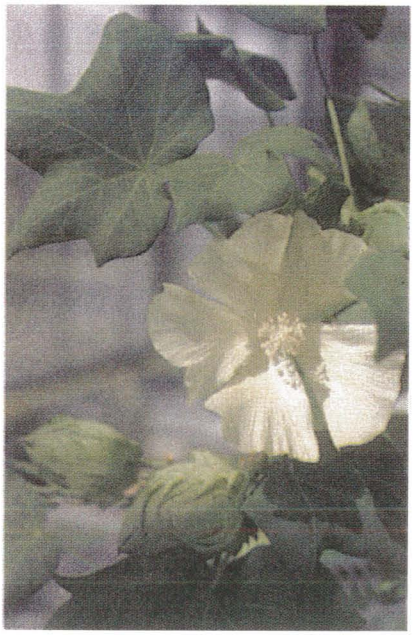




12. Histological sections of somatic embryos of cotton.

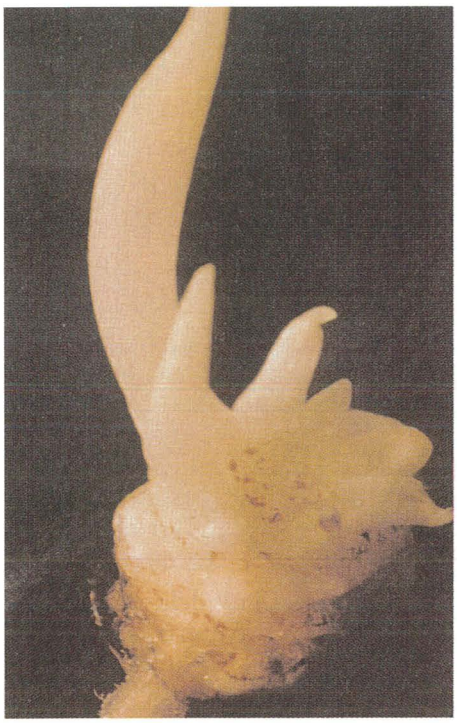
14. Young plantlets of cotton.

13. Somatic embryos of cotton: on the left, embryos expressing the gus *gene.*

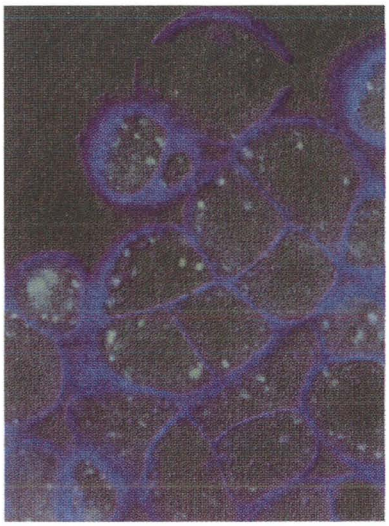


15. Transgenic cotton plants in the greenhouse.

16. Flower and bolls of a transgenic cotton plant.



17. Banana meristem proliferation.



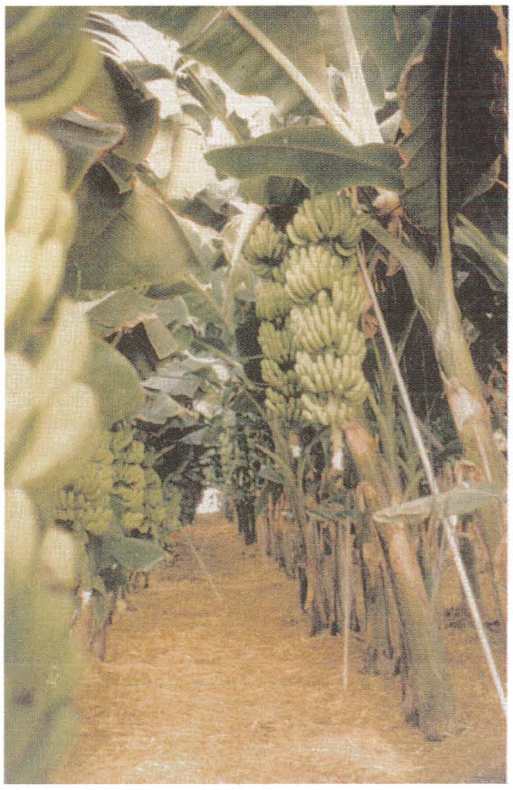
18. Banana embryogenic cell suspension stained with calcofluor.

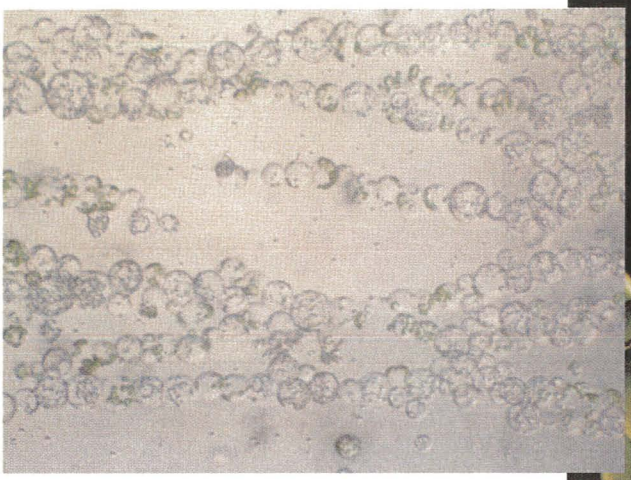
19. Banana somatic embryo germination.

20. Banana embryogenic cell suspension.

21. Banana plantlet nursery.

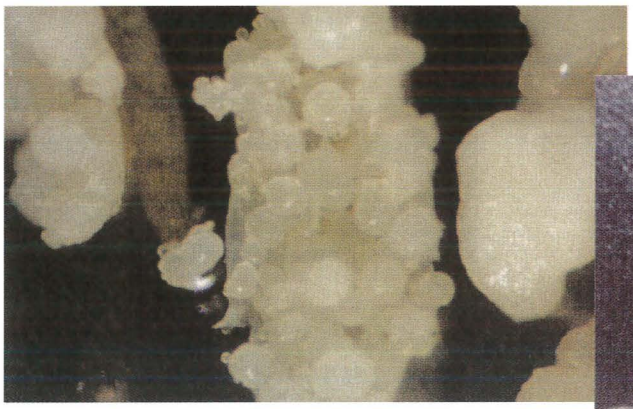
22. Banana field from vitroplantlets.



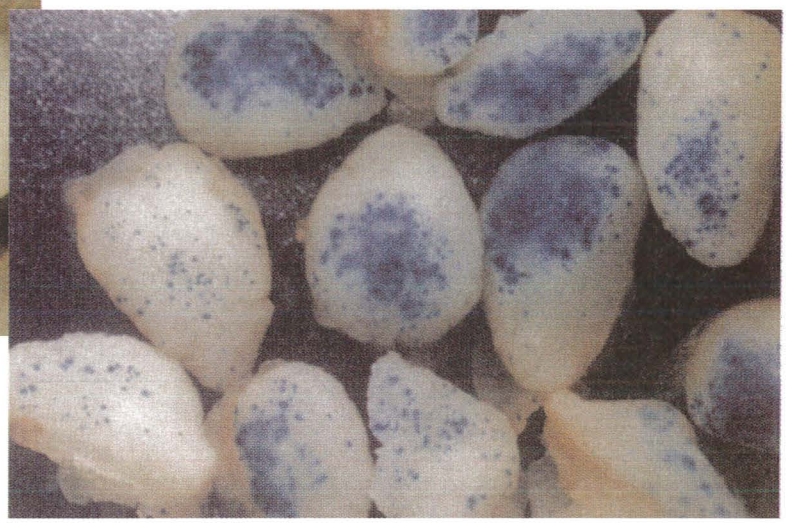


25. *Electrofusion of* Citrus *protoplasts.*

24. Citrus deliciosa *somatic embryo germination.*



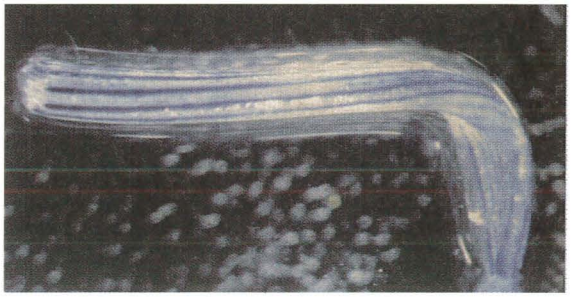
23. *Rice androgenesis.*



26. *Transient expression of* gus *gene on rice scutellum.*

27. *Stable rice transformed plants.*

28. *Stable* gus *expression with a phloem-specific promotor*



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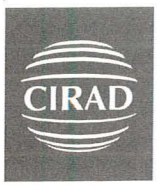

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